The influence of the gut microbiota on host mucosal immunity and its impact on gastrointestinal inflammation

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Summary

In healthy individuals the intestinal microbiota, a complex microbial ecosystem, and the host maintain a mutually beneficial relationship. The microbiota contributes significantly to fundamental physiological functions of the host such as the degradation of complex food ingredients, colonization resistance against pathogenic bacteria and the development of host immune system. A balanced composition of the microbiota is crucial to provide its beneficial functions to the host's health. In contrast, alterations at the overall phylum level, the addition or lack of specific members or of particular functions of the gut microbiota have been associated to worse or recover disease outcomes. Specifically, studies have demonstrated that alteration in the composition of gut microbiota can dramatically affect host immunity and are frequently associated to common gastrointestinal diseases. Therefore it is essential to investigate and identify the key players that tip the balance between pathogenic or homeostatic host-microbiota interactions to eventually develop novel microbiome-centered therapeutics to modulate inflammatory diseases.

Here we demonstrated that alterations in gut microbial communities are able to directly enhance the disease severity in a mouse model of human inflammatory bowel disease (IBD). Specifically, we unveiled that distinct microbial communities utilized opposing arms of host immunity to promote disease severity. While one community required microbiota-specific CD4+ T cell responses, another community triggered severe intestinal inflammation even in the absence of adaptive immunity. Intriguingly, an overexpansion in the Proteobacteria over the Firmicutes phylum was associated to pathogenic CD4+ T cell response.

Next, we elucidated beneficial CD4+ T cell responses driven by specific commensals during intestinal inflammation. Specifcally, we explored a previously reported immunomodulatory bacteria, segmented filamentous bacteria (SFB) and its effect on *Salmonella* induced gastroenteritis. While this bacterium is known to induce proinflammatory cells, SFB specific CD4+ T cells served as "innate-like" source of antibacterial cytokines rapidly after infection. Importantly, SFB colonized mice displayed reduced pathogen invasion in the cecum suggesting that modulation of CD4 T cells by SFB is associated with improved immune defense.

Together our studies demonstrate that the intestinal microbiota can directly influence host immunity to exert both pathogenic and beneficial disease outcome. Further studies are needed to explore specific interactions among distinct microbial members and host factors to drive different disease pathogenicity. Elucidation of similar microbiota induced effects in human may help to develop personalized therapies.

Zusammenfassung

Die intestinale Mikrobiota ist ein komplexes mikrobielles Ökosystem, das mit dem Wirt in einer gegenseitigen positiven Wechselwirkung steht. Die Mikrobiota spielt eine entscheidende Rolle in der Physiologie des Wirts; insbesondere in der Nahrungszersetzung, der Kolonisierungsresistenz gegenüber pathogenen Bakterien und der Entwicklung des Immunsystems. Eine günstige Zusammensetzung der Mikrobiota ist wichtig, um nützlichen Funktionen für die Gesundheit des Wirts zu garantieren. Im Gegensatz dazu werden mikrobielle Veränderungen und die Zugabe oder das Fehlen spezifischer Mitglieder bzw. bestimmter Funktionen der Darmmikrobiota mit einer Verschlechterung oder Verbesserung der Krankheit assoziiert. Studien haben gezeigt, dass eine Veränderung der Zusammensetzung in der Darmmikrobiota die Wirtsimmunität drastisch beeinflussen kann und häufig mit Magen-Darm-Erkrankungen assoziiert ist. Daher ist es von Bedeutung, die wichtigsten Akteure, die das Gleichgewicht zwischen pathogenen oder homöostatischen Wechselwirkungen mit der Mikrobiota beeinflussen können, zu identifizieren und zu untersuchen. Dies ist wichtig und vielversprechend, um letztendlich neue Mikrobiom-basierte Therapeutika zu entwickeln, die entzündliche Erkrankungen modulieren können.

In dieser Dissertation konnte gezeigt werden, dass Veränderungen in der Darmmikrobiota in Mäusen einen direkten Einfluss auf die Schwere von chronisch-entzündlichen Darmerkrankungen (CED) haben. Außerdem konnte nachgewiesen werden, dass verschiedene mikrobielle Gemeinschaften gegensätzliche Signale der Wirtsimmunität nutzten, um den Schweregrad der Krankheit zu regulieren. Während eine Gemeinschaft von Bakterien spezifische CD4+ T-Zellantworten benötigte, um eine schwerwiegende Darmentzündung auszulösen, konnten diese von einer anderen Gemeinschaft auch in Abwesenheit einer adaptiven Immunantwort geschehen. Darüber hinaus war eine Überexpansion an Proteobakterien mit einer pathogenen CD4+ T-Zellreaktion assoziiert.

Zudem wurden nützliche CD4+ T-Zellantworten entdeckt, die durch spezifische kommensale Bakterien während der Darmentzündung angeregt werden. Insbesondere wurde die Wirkung eines zuvor beschriebenen immunregulierenden Bakteriums, das „Segmentierte filamentöse Bakterium“ (SFB), auf *Salmonella*-induzierte Gastroenteritis untersucht. Es war bereits bekannt, dass dieses Bakterium pro-inflammatorische Zellen induziert, SFB-spezifische CD4+-T-Zellen wurden hier als neue "angeborene“ Quelle von schnell induzierten antibakteriellen Zytokinen während der Infektion identifiziert. SFB-kolonisierte Mäuse zeigten eine reduzierte Pathogeninvasion im Cecum zeigten, was. Daraufhin deutet, dass die Modulation von CD4-T-Zellen durch SFB mit einer verbesserten Immunabwehr verbunden ist.

Zusammenfassend konnten gezeigt werden, dass die intestinale Mikrobiota die Wirtsimmunität direkt beeinflussen kann und somit Krankheitsverläufe verbessert oder verschlechtert. Weitere Untersuchungen sind erforderlich, um spezifische Wechselwirkungen zwischen verschiedenen mikrobiellen Mitgliedern und Faktoren des Wirts zu erforschen, um eine unterschiedliche Krankheitspathogenität zu bewirken. Die Aufklärung ähnlicher Mikrobiota-induzierter Effekte bei Menschen kann dazu beitragen, personalisierte Therapien zu entwickeln.

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List of Abbreviation

AhR	aryl hydrocarbon receptor
AMP	antimicrobial peptide
ASF	altered Schaedler flora
BFP	blue fluorescent protein
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
CD	Crohn's disease
DC	dendritic cell
DNA	deoxyribonucleic acid
DSS	dextran sulfate sodium
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FMT	fecal microbiota transplantation
Foxp3	forkhead box P3
GF	germ-free
GFP	green fluorescent protein
GI	gastrointestinal
GWAS	genome-wide association study
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IEL	intraepithelial lymphocyte
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
KC	keratinocyte chemoattractant
LIX	lipopolysaccharide-induced CXC chemokine
LPL	lamina propria leukocytes
MIP	macrophage inflammatory protein
MYD88	myeloid differentiation primary response gene 88
NK	natural killer

NLRP	NOD like receptor protein
NMDS	nonmetric multidimensional scaling
NOD	nucleotide-binding oligomerization domain
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PRR	pattern recognition receptor
RAG	recombination activating gene
RFP	red fluorescent protein
RNA	ribonucleic acid
RORC	RAR-related orphan receptor C
RORγ	RAR-related orphan receptor gamma
ROS	reactive oxygen species
SCFA	short-chain fatty acid
SFB	segmented filamentous bacteria
sIgA	secretory immunoglobulin A
SNP	single nucleotide polymorphism
SPF	specific pathogen free
TBX21	T-box transcription factor
TCR	T-cell receptor
TGFβ	transforming growth factor β
TLR	toll like receptor
TNF	tumor necrosis factor
UC	ulcerative colitis
WT	wild type

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CHAPTER 1

General Introduction

The Gastrointestinal (GI) tract of all vertebrates harbors diverse communities of microorganisms consisting of large numbers of bacteria, fungi, parasites and viruses commonly known as gut microbiota. In healthy individuals the intestinal microbiota and host maintain a symbiotic balance where the microbiota contributes to diverse physiological processes in the host and the host in turn provides a nutrient rich niche for the microbes. In recent years our knowledge about human gut microbiome have improved significantly and studies have discovered profound effect of the composition of the intestinal microbiota in human health. Substantial advances in culture independent sequencing based techniques have allowed insights into the compositional diversity of the human gut microbiota as well as contributions of diet, geography and individual variability [1–3].

Alterations in the gut microbial composition can result from changes in host physiology due to environmental challenges and/or genetic defects. Numerous studies have associated changes in the gut microbiota composition with various disorders ranging from gastrointestinal diseases to systemic diseases such as obesity, allergy and neurodegeneration. Based on different disease models both beneficial and harmful effects of microbiota on disease outcome have been described. However, some important aspects of the influence of the intestinal microbiota to host pathophysiology still remains to be elucidated: 1. Can alteration in gut microbiota composition or a specific member of the commensal bacteria directly affect inflammatory disorder? 2. What specific host immune response mediated by the intestinal microbiota can result in beneficial or harmful outcome in different disease systems? Investigating these important aspects of the microbiota study can help to target microbiota or microbiota induced pathways to advance personalized treatment towards microbiota-mediated diseases.

1.1 Microbiota-host interaction in the gastrointestinal tract during homeostasis

Starting with birth, the mucosal surfaces of the mammalian GI tract face a continuous challenge of constant exposure to complex microbial communities. More than 100 trillion microorganisms, comprising over 500 species of bacteria harbor an adult human. In healthy individuals, Gram-negative Proteobacteria and Bacteroidetes, and Gram-positive Firmicutes, are the major phyla among intestinal eubacteria, whereas methanogens are the predominant intestinal archaea.

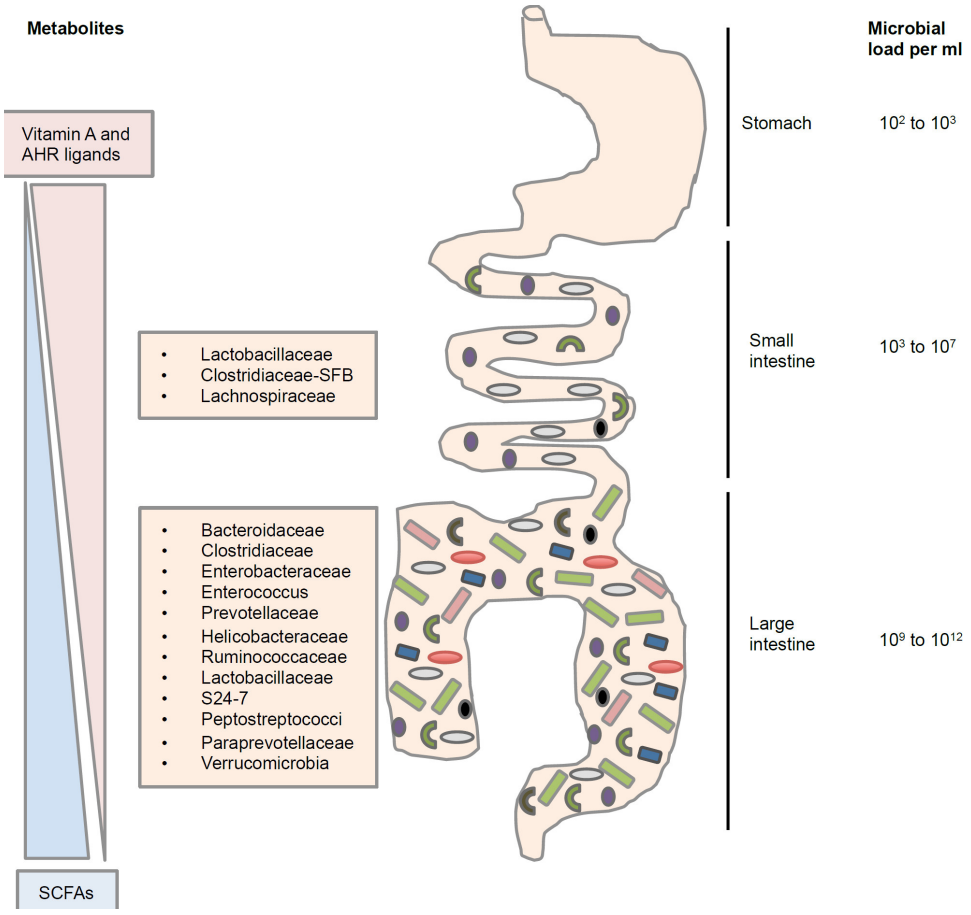

Figure 1.1 Distribution of microbiota and metabolites throughout the intestine.

The numbers and diversity of the gut microbiota increase towards the distal GI tract, ranging from 10^2-10^3/ml in stomach upto 10^{12}/ml in colon. Availability of different sources of nutrients vary greatly throughout the GI tract, i.e. a decrease in the level of vitamin A and an increase in short chain fatty acids (SCFAs) level can be observed towards distal part of the GI tract.

Diversity and concentrations of colonized bacteria vary according to anatomic location as well as availability of nutrients and increases along with the distal part of the intestine (Figure 1.1). Large fractions of these intestinal bacteria are anaerobic and very difficult to grow under laboratory culturing conditions. However, recent advancement in sequencing techniques has allowed us not only to gain insight their detailed composition using 16S ribosomal RNA based sequencing, but also characterize their functionalities by analyzing the genomic composition using shot gun metagenome-sequencing.

The primary function of gut microbiota is to enhance host digestive efficiency. Fermentation of complex polysaccharides and oligosaccharides by colonic microorganisms results in the synthesis of SCFAs such as butyrate, propionate and acetate, which are rich sources of energy for the host [4]. Gut microbiota also contributes to lipid metabolism and hydrolysis. Protein metabolizing machinery that function via microbial proteinases and peptidases in tandem with human proteinases are also enriched in the gut microbiota. Another major metabolic function of the microbiota is synthesis of vitamin K and several components of the vitamin B2 family. Distinct members, i.e. *Bacteroides intestinalis*, *B. fragilis*, *Escherichia coli* have the capacity to dehydrate and deconjugate the primary bile acids and convert them into secondary bile acids in human colon [5]. Besides significant contributions to host metabolic functions, the gut microbiota also plays critical roles in educating the host immune system, which will be discussed later in detail.

1.1.1 Mucosal immune system maintaining microbial balance

In past models, all microorganisms were viewed as potential pathogens that could cause and propagate infectious diseases and, as a field, immunology was built around the paradigm that to preserve homeostasis the host immune system should recognize and eliminate these intruders (non-self) while tolerating self-molecules. However, animal and plant species have shown persistent association with obligate and facultative symbionts, which requires refining our idea of beneficial cooperative relationships between bacteria and

their eukaryotic hosts. Therefore, a new paradigm was proposed suggesting that the immune system has evolved to accommodate colonization by symbiotic bacterial communities of increasing complexity while retaining the capacity to fight pathogens. Development of host immune factors to maintain homeostatic microbial load in the GI tract has been extensively studied and some of the important host factors will be discussed below.

Bacteria-host epithelium contact minimization:

Minimization of the contact between intestinal bacteria to its host epithelium is one of the first stages to maintain microbial balance in the gut (Figure 2A). The mucus layer on top of the host epithelium specifically in colon comprises a variety of mucin glycoproteins secreted by goblet cells, forming two structurally distinct layer, one dense inner layer devoid of bacteria and the other loose outer layer where commensal bacteria can reside [6]. Epithelial barrier also protects host from huge bacterial load by epithelial cells secreted antimicrobial proteins (AMP). Most of these antimicrobial proteins i.e. defensins, cathelicidins, C-type lectins kill bacteria directly through enzymatic attack or disruption of bacterial inner membrane and some subset including lipocalin 1 results deprivation of essential metals like iron for the bacteria [7]. Secretory immunoglobulin A (IgA) plays also a significant role to limit bacterial association with the intestinal epithelial cell surface [8]. Transcytosed IgA secreted from plasma cells binds to bacteria on the luminal side limiting epithelial association of bacteria and preventing bacterial penetration of host tissues [9].

Controlling of penetrated bacteria via immune responses:

Even after a strict control at the epithelial barrier some intestinal bacteria can penetrate to mucosal tissue where distinctive immune mechanisms play role to detect and kill penetrated bacteria (Figure 2A). Lamina propria macrophages provide such mechanism via phagocytosis of bacteria followed by killing via AMPs and reactive oxygen species (ROS) [10]. In addition, CD4+ T cells specifically Foxp3+ regulatory T cells (Tregs) are an essential component to maintain tolerance towards environmental antigens including

commensal bacteria. Tregs can control the balance of bacterially induced effector cells like Th1 cells and in addition regulatory cytokines secreted by Tregs such as IL-10 and TGF-β are also equally important to maintain intestinal homeostasis. Notably, spontaneous inflammation has been observed in mice deficient in IL-10 [11] or TGF-β [12]. Similarly, Th17 cells also have been reported to regulate normal intestinal microbiota. Some other subsets of host immune system i.e. intraepithelial CD8aa+ T cells and natural killer (NK) cells are also being studied to associate with maintaining host-microbiota balance. These studies together strongly suggest that numerous host factors are engaged to strictly regulate the microbial load in the GI mucosa and breaches in such regulatory mechanisms may allow microbial antigens to penetrate both locally and systemically and affect host health.

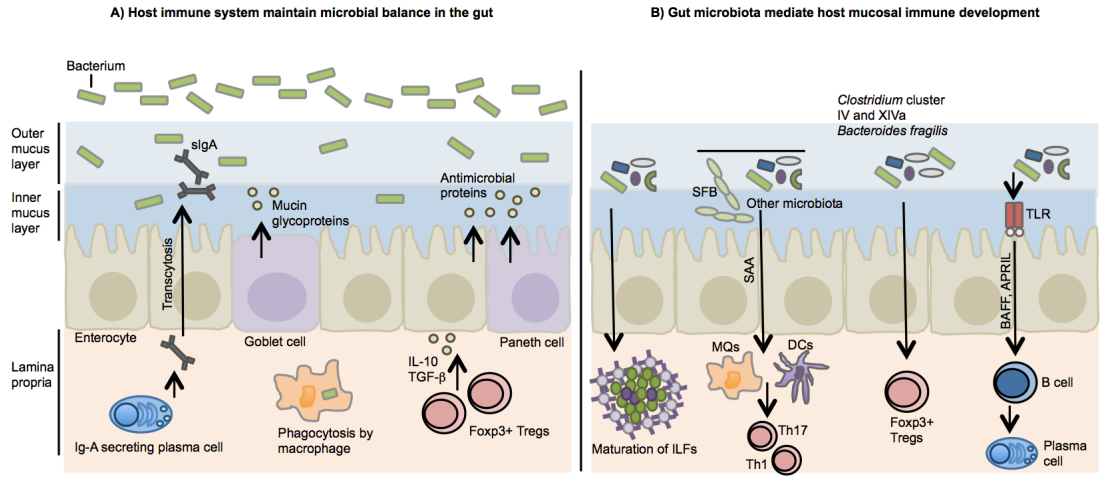

Figure 1.2 Host-microbiota interaction in the intestine during homeostasis.

A) Host immune system maintains the microbial load at the mucosal surface of the intestine. Inner thick mucus layer halts bacterial entry, AMPs produced by different cell subsets in the epithelium kills penetrated bacteria and different host immune cells provide protection via sIgA secretion, phagocytosis and Treg generation.

B) Immune system development in host is strongly modulated by gut microbiota. Intestinal bacteria helps maturation of isolated lymphoid follicles (ILFs), generation of IgA secreting plasma cells. Some specific members of bacteria helps to develop different subsets of immune cells, i.e. SFB mediated Th17, *Clostridium* spp. mediated Treg development.

1.1.2 Intestinal bacteria modulating mucosal immunity and functionality

As important it is for the host to maintain commensal load to the mucosal surface, beneficial functions provided by balanced microbiota are similarly important for proper development of host mucosal immunity (Figure 2B). Continuous exposure to different bacteria throughout life provides benefits from lymphoid tissue development to maturation or educating immune system. Studies performed in germ free (GF) animals have demonstrated influence of microbiota to maintain host immunity, however, only a handful studies could probe direct association of specific microbial species involved in modulating particular host immunity.

Development of gut associate lymphoid tissue (GALT), an important immune structure for antigen uptake and presentation, significantly regulated by intestinal bacteria. Although in fetus lymphoid tissue inducer (LTi) cells promote development of Peyer's patches, the maturation of isolated lymphoid follicles (ILFs) and crypt patches requires stimulation by the gut microbiota [13,14].

Besides morphological tissue development of immune system, gut microbiota also plays significant role for the development and accumulation of distinct subsets of leukocytes both locally and systemically. Intestinal bacteria driven signals have regulatory influences on intestinal antigen presenting cells (APCs). Dendritic cell (DC) populations in the intestine are reduced in GF animals and has shown to recruited upon monoassociation with *E. coli* [15,16]. While numbers of monocytes/macrophage were normal in intestinal tissue, systemic monocyte/macrophage numbers were reduced in animals reared under GF conditions and colonization with *Lactobacillus acidophilus* and *L. reuteri* could recover such defect [17]. Moreover, some studies have suggested that microbiota is important for proper differentiation of innate lymphoid cells (ILCs) to produce IL-22 [18,19].

Contributions of the gut microbiota and specific members to modulate host adaptive immunity have been studied extensively. Gut-specific B cell responses are intimately related to microbiota. In the intestine plasma cells derived from B cells produces polymeric IgA at high concentrations, which is

then transported and released into the intestinal lumen as secretory IgA (sIgA) [20]. In GF mice, numbers of IgA-producing cells in the intestine are significantly reduced [21]. One important mechanism thought to influence IgA production is bacterial recognition through MYD88 in follicular DCs [22]. Notably, DC recognition of flagellin derived from commensal bacteria promotes retinoic acid synthesis facilitating differentiation of IgA producing B cells [23,24]. Furthermore, in lamina propria DCs, intestinal bacteria enhance expression of factors that are involved in induction of IgA+ B cells, i.e. tumor necrosis factor (TNF), inducible nitric oxide synthase (iNOS), B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) [25,26].

Adaptive T lymphocytes specifically CD4+ helper T (Th) cells are crucial for host immune responses both during health and diseases. Distinct lineages of Th subsets with potentiality of different immunoregulatory cytokine secretion are closely regulated by the gut microbiota. One of the best examples is the generation of intestinal Th17 cells, a subset of activated CD4+ T cells characterized by production of IL-17A, IL-17F and IL-22 [27], by particular species of Clostridia-related bacteria, called segmented filamentous bacteria (SFB) in mice [28–30]. Adhesion of SFB to the intestinal epithelial cell (IEC) is indispensible for Th17 cell development [31]. Although SFB was not observed in human, a mixture of 20 bacterial strains isolated from human patients, able to induce Th17 cells in mouse colon, also demonstrated IEC-adhesive characteristics [31]. SFB colonization results in robust IL-22 secretion by type 3 ILCs that induces serum amyloid A protein (SAA) production by IECs in a Stat3-dependent manner [32]. The aforementioned study also showed that early after SFB colonization RORγt+ T cells were induced in mesenteric lymph nodes followed by distribution across different parts of GI tract and continuous IL-17A production however was only restricted in ileum.

Intestinal bacteria also regulate another subsets of CD4+ Th cells, Foxp3+ Tregs that are strictly required to maintain gut homeostasis. Peripherally derived Treg cells are greatly decreased in the colonic lamina propria of GF mice [33,34]. Several studies have shown colonization of GF mice with distinct groups of commensal bacteria can recover accumulation of IL-10 producing Treg cells in colon, i.e. a mixture of 46 Clostridium spp. cluster IV and XIVa

strains; altered Schaedler flora (ASF), a cocktail of eight defined commensal bacteria; or the human commensal bacterium *Bacteroides fragilis* [33–35]. However, the mechanism behind bacterially induced Treg development still remains to be elucidated.

Finally the microbiota also contributes to the proliferation and function of different other T cell subsets. For example, in GF mice numbers of TCR$\alpha\beta$+ intra epithelial lymphocytes (IELs) are reduced and colonization with SFB and Clostridium spp. induces accumulation of this IEL subset in small and larger intestines respectively [36]. Moreover, intestinal bacteria have been shown to particularly expand IL-1R1 expressing IL-17 producing $\gamma\delta$ T cells in conventionally raised mice [37].

1.2 Host-microbiota interplay during GI inflammation

Gastrointestinal diseases are a substantial burden and responsible for approximately eight million deaths per year worldwide and one million deaths each year across Europe. Diarrheal disease is the second leading cause of death among child <5 years old, with the majority of these deaths occurring in Africa. Gastrointestinal-related cancers are responsible for nearly 40% of all cancer-related deaths with a toll of nearly 3 million deaths per year estimated in 2008. The incidence and prevalence of many GI diseases are highest amongst the very young and the elderly also causing significant morbidity and healthcare costs. GI tract encounter different forms of challenges though food, environment, habitat resulting different forms of infectious and autoimmune diseases. Many aspects of digestive diseases are still poorly understood e.g. the involvement of the gut microbiota. In the following I will discuss two very common forms of autoimmune and infectious disease of the GI tract and specifically the interplay of the host and the intestinal microbiota during such inflammatory disorders.

1.2.1 Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is characterized by chronic relapsing inflammatory disorders of small and/or large intestine resulting in recurrent diarrhea and abdominal pain. There are two main clinicopathological subtypes of IBD- Crohn's disease (CD) and ulcerative colitis (UC). CD can affect any part of the GI tract most commonly the terminal ileum and colon and is microscopically characterized by transmural inflammation, thickening of submucosa, fissuring ulceration and granuloma formation in a discontinuous pattern. UC on the other hand is restricted to colon and rectal area and forms continuous superficial inflammatory changes in the mucosa and submucosa with cryptitis and crypt abscesses [38]. For IBD development family history is an important risk factor, with a higher incidence in early adult life. A high prevalence of IBD is observed in industrialized countries, with the highest reported incidences in northern Europe and North America. An estimated 2.5-3 million of Europeans suffers from IBD with a direct healthcare cost of 4.6 - 5.6 billion Euros per year. A direct etiology for IBD development is still unknown. However, association of several risk factors have been studied and reporter. Different environmental factors, such as smoking, diet, drugs, geography, social stress, and psychological element have been reported as considerable risk factors [39]. However, in most incidences IBD is thought to occur in genetically susceptible individuals by an abnormal immune response against the microorganisms of the intestinal flora. Therefore, more and more careful investigations are being carried out to understand the interplay between host immunity and microbiota in the initiation of IBD. Common IBD therapies include interventions on life-style habits and medical and surgical treatments, where medical management involves use of corticosteroids, immunosuppressant agents and biological therapies [38]. However, recent studies are proposing towards targeting microbiota and microbiota-mediated host immune pathways to control IBD.

Host immune factors responsible for IBD development:

Genome-wide association studies (GWAS) that identify single nucleotide polymorphisms (SNPs) have contributed significantly to identify host genetic factors to IBD. Until now 163 hosts gene loci associated to IBD were identified [40,41]. Gene loci observed from GWAS and other coherent studies have highlighted a significant involvement of distinct innate and adaptive immune pathways to develop IBD. While most studies in the last two decades have focused on the role of abnormal adaptive immune responses to IBD development, recent studies have found that many IBD patients display dysfunctional mucosal innate immunity.

Innate immunity provides a first line of defense, e.g. the epithelial barrier provides a first physical barrier towards bacterial and food antigens. In patients with CD and UC, a defective epithelial barrier and an increased intestinal permeability have long been observed [42]. Particularly in UC patients several SNPs, i.e. in *HNF4A*, *CDH1*, *LAMB1* and *GNA12*, suggesting that epithelial barrier defects are found to be associated to disease susceptibility [43,44]. In CD patients on the other hand a defective antimicrobial peptide expression have been observed. Compared to UC, inflamed colon of CD patients showed reduced induction of β-defensins *HBD2*, *HBD3* and *HBD4* [45]. Defects in microbial antigen sensing by innate immune cells, such as macrophages and DCs are strongly associated to IBD formation. Different pattern recognition receptors (PRRs) of host immune cells recognize microbial antigens. PRRs include trans-membrane Toll-like receptors (TLRs) and intracytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). *NOD2* was the first susceptibility gene identified to confer increased risk to CD [46,47]. An important innate immune process of controlling intracellular bacterial invasion is autophagy. Mutations in genes involved in autophagy, such as, *ATG16L1* and *IRGM* have been associated to CD [48,49]. Moreover, a key cytokine, IL-23, orchestrating the crosstalk between innate and adaptive immunity plays a central role to regulate early immune responses to microbes. SNPs in *IL23R* gene that encodes specific subunit of IL23 receptor have been identified in different independent cohorts of IBD patients [50]. Some other IBD risk variant genes such as *STAT3* and *JAK2*,

involved in IL-23 signal transduction are also shown to be associated with IBD [51]. Neutrophils, short-lived innate immune cells have ability to destroy extracellular and intracellular invading pathogens. Accumulation of three fold increased numbers of activated neutrophils have been observed in the blood of UC patients [52]. Blockade of neutrophil significantly attenuated inflammation in murine experimental colitis [53,54].

Adaptive immune system is highly specific and provides long lasting immunity. Dysregulation of adaptive immune cells, specifically T cell responses resulting excessive release of different pro-inflammatory cytokines and chemokines have been associated to CD and UC. Classically, it has been considered that CD is characterized by Th1 immune responses, while UC is mediated by Th2 responses [55]. Abnormal Th1 immune responses, triggered by release of IL-18 and IL-12, is thought to cause intestinal inflammation in CD [56,57]. It has also been demonstrated that activated T cells from CD mucosa release more IFN-γ than T cells from UC patients or controls, whereas UC patients produce more IL-5 [58]. Also NK T cells in UC patients release higher amounts of Th2 cytokine IL-13 than from controls or CD patients [59,60]. Notably, tumor necrotic factor (TNF)-α, a pro-inflammatory cytokine secreted by Th1 and also by mucosal macrophages, plays a crucial role in IBD pathogenesis. Impressive results have been observed in clinical trials with biologic agents targeting TNF-α [61]. Furthermore, Th17 cells, another important T cell subsets, are induced by a combination of IL-6 and transforming growth factor (TGF)-β. High transcript levels of IL-17A secreted by Th17 cells have been detected in CD and UC mucosa in comparison to normal gut, and also overexpression of IL-17A by immunohistochemistry has been observed in lamina propria of IBD patients [62,63]. Additionally, IL-21, an IL-2 related cytokine is upregulated in inflamed mucosa of IBD patients and is produced by Th17 cells [64,65]. Distinct T cell subsets play crucial role to maintain homeostatic balance of host immune system. Foxp3 expressing regulatory T cells are important to maintain gut homeostasis and to suppress abnormal immune responses by producing anti-inflammatory cytokines IL-10 and TGF-β. Tregs are depleted in peripheral blood of active IBD patients compared to quiescent IBD patients and controls [66]. Besides T-cell dependent influence of IBD development, B-cell defects

leading to colitis have also been demonstrated in some experimental studies. Moreover, recent GWAS of deficiency in IgA, produced by B cells, showed genes also implicated in IBD, i.e. *ORMDL3*, *REL* and *PTPN22* [67]. Together these aforementioned studies strongly support the involvement of host immune system for IBD development. However, targeting these host immune factors to ameliorate IBD shows variable outcomes and still needs to be studied in detail what other factors are interplaying to develop such complex pathology.

Contribution of gut microbiota to IBD:

Many studies have demonstrated a correlation between alterations in the gut microbiota and IBD development. Notably, studies performed in animal colitis models under GF and other gnotobiotic microbiota conditions have shown a direct influence of certain microbial species to cause enhancement of colitis severity by modulating host immune and/or metabolic pathways (Figure 3A). However, which specific intestinal bacteria directly regulate development and progression of IBD in human patients still remains to be investigated. Compared to the healthy intestine, an overall decrease in resident Firmicutes and over-representation of certain Bacteroides and/or Proteobacteria have been observed in IBD patients. Clostridium clusters XIVa and IV among Firmicutes, are largely underrepresented in IBD patients [68–70]. A significant reduction of *Faecalibacterium prausnitzii* abundance is observed in patients with active IBD compared to those in remission [71] and also in patients with recurrent IBD [72] suggesting a anti-inflammatory role of *F. prausnitzii*. Presence of *F. prausnitzii in vitro* resulted in production of anti-inflammatory IL-10 and inhibited secretion of pro-inflammatory IL-12 and IFN-γ in human PBMC [72]. *F. prausnitzii* is able to metabolize diet-derived polysaccharides and other substrates to produce butyrate [73,74], a major source of energy for colonic epithelial cells and mucosal health. Microbiota-produced butyrate has also been shown to induce Treg development [75,76]. Abundances of other butyrate-producing bacteria, i.e. *Roseburia hominis* and *Eubacterium rectale* are similarly reduced in IBD patients [77,78]. Moreover reduction in *Bifidobacterium adolescentis*, a folate-producing bacterium was observed in IBD patients

[68,70,79]. Folate is known to enhance Treg survival and thereby diminish intestinal inflammation [80]. Expansion of Proteobacteria phylum in IBD patients was revealed by several studies [81–83]. Of the Proteobacteria, one of the most studied and reported group is Gammaproteobacteria, specifically, *Escherichia coli*. Pathogenic *E.coli* strains accumulate in intestinal stool and mucosa of IBD patients, especially CD [84–86]. Adherent-invasive *E.coli* (AIEC) strains from IBD patient have been isolated and shown to adhere and invade intestinal epithelial cells and colonize the ileal mucosa of CD [87]. AIEC also replicate in macrophages and stimulate TNF-α production from macrophages. Certain Gammaproteobacteria such as, *Klebsiella* spp., *Pseudomonas* spp., and *Salmonella* spp. have also been implicated in IBD [88,89]. Deltaproteobacteria comprise sulfate-reducing bacteria and the abundances of these bacteria are also increased in IBD patients [90–92]. Sulfate-reducing commensal *Bilophila wadsworthia* increases due to excessive dietary taurocholic acid and can cause Th1 mediated colonic inflammation in *IL-10*$^{-/-}$ mice [93]. Moreover, abundances of some Epsilonproteobacteria i.e. *Campylobacter concisus* and *Campylobacter jejuni* are higher in intestinal biopsies or fecal samples of IBD patients [94,95]. These *Campylobacter* spp. attach to intestinal epithelial cells to secret IL-8 and IL-8 recruits neutrophils, DCs and macrophages. These recruited cells then interact with internalized bacteria resulting secretion of pro-inflammatory cytokines, IL-1, IL-6, IL-12 and TNF-α [94,96,97]. Taken together, both presence and absence of distinct gut commensals is positively correlated to induce intestinal inflammation. It is therefore important to assess whether these commensals can induce distinct pathway to result similar inflammatory outcomes.

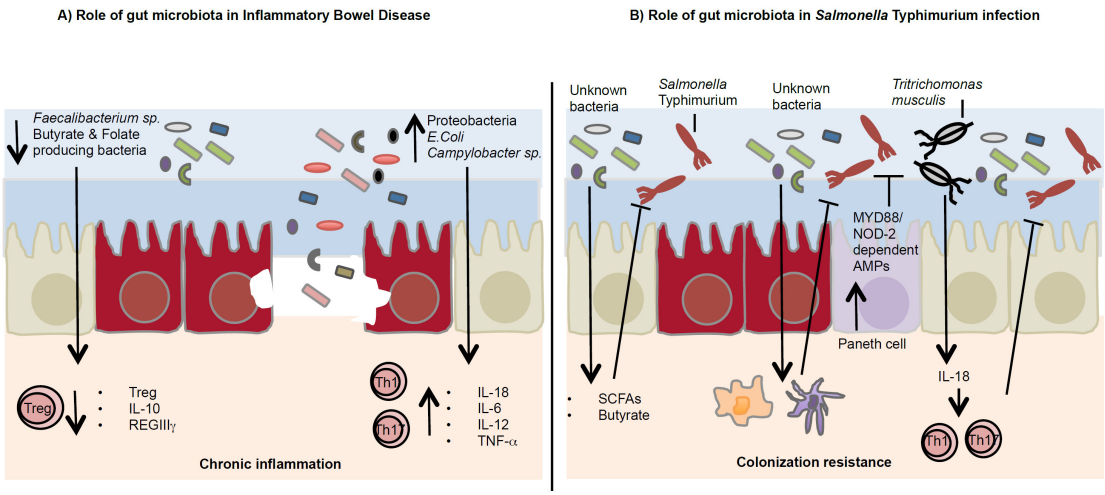

Figure 1.3 Role of gut microbiota during gastrointestinal inflammation.

A) Studies have demonstrated that the gut microbiota contribute to IBD development. Decrease in *Faecalibacterium* sp. or other butyrate and folate producing bacteria result in decrease of regulatory T cells thereby enhance colitis development. Increase in members of Proteobacteria result in increased production of proinflammatory cytokines i.e. IL-6, IL-12, TNF-α etc. by Th1 and/or Th17 cells, which leads to formation of chronic inflammation. B) During *Salmonella* induced gastroenteritis gut microbiota provides colonization resistance via different mechanisms. SCFAs and butyrate produced by intestinal bacteria control *Salmonella* invasion. Microbiota induced development of phagocytic immune cells and AMP secretion by paneth cells can act on pathogen killing. Commensal protozoa *Tritrichomonas musculis* promotes IL-18 secretion followed by generation of Th1 and Th17 immunity against *Salmonella* pathogen to provide protection.

1.2.2 *Salmonella* induced intestinal inflammation

Similar to IBD, also during infectious diseases in the GI tract host immunity and the microbiota act in concert promoting intestinal inflammation. Infections caused by *Salmonella* spp. result both intestinal and systemic diseases in human and animal hosts and are crucial public health priority worldwide [98–100]. *Salmonella* spp. are Gram-negative facultative intracellular pathogens belonging to the family Enterobacteriaceae. Depending on bacterial serovar and underlying host susceptibility *Salmonella* spp. cause different forms of clinical diseases [101]. Three distinct categories of *Salmonella* infections have been described:

i) *Salmonella* gastroenteritis, a localized intestinal infection, mainly results from ingestion of contaminated food products [102,103]. An

approximate of 30-50% of food-borne infections is attributed to *Salmonella* serovars in the USA [104,105]. Common symptoms include fever, diarrhea and cramping, however, most healthy individuals recover without treatment within 4-7 days.

ii) Typhoid fever, a systemic *Salmonella* infection of healthy host, mainly caused by *Salmonella enterica* serovars Typhi and Paratyphi. An approximate of 21.7 million cases and 217,000 deaths annually in developing nations are caused by Typhoid fever [106]. This disease is characterized by fever and hepatosplenomegaly with subsequent intestinal hemorrhage or perforation. A common way of prevention is vaccination.

iii) Non-typhoidal Salmonellosis (NTS), a systemic infection of an immune-compromised host, results in fatal infections, which can be disseminated without gastrointestinal symptoms and can be transmitted by human-to-human contact. An increased association of NTS dissemination has been observed in HIV-infected individuals in Asia and Africa [107].

Animal models, especially mouse models are being extensively used to understand pathogenesis, host response and involvement of the gut microbiota in *Salmonella* infections. Oral infection with *S.* Typhimurium in genetically susceptible mouse strains leads to spread of pathogen to systemic sites via gut-associated lymphoid tissues, which is similar to human typhoid fever [108]. On the other hand, *S.* Typhimurium causes chronic infection of systemic organs in genetically resistant inbred mouse strains, which provides a useful animal model for persistent systemic infection [109]. However, *Salmonella* cannot colonize the mouse intestine in all these typhoid fever models, which makes it difficult to study *Salmonella* driven enterocolitis in mouse models. This has been overcome by study demonstrated, a high-dose of oral streptomycin treatment of mice results efficient colonization of *S.* Typhimurium in large intestine and triggers severe acute inflammation of cecum and colon [110,111].

Host immune responses to *Salmonella:*

The primary site of *Salmonella* infection are specialized microfold, or M cells in Peyer's patches [112]. M cells outside of Peyer's patches and direct sampling of *Salmonella* by phagocytes in the lamina propria also contribute to the entry of the pathogen. *Salmonella* express a type-III secretion system, which is necessary for its M-cell invasion [113]. *Salmonella* encounters various phagocyte populations after invasion into the host tissues. The pathogen can survive and replicate in macrophages after phagocytosis. However, by producing lysosomal enzymes, reactive oxygen intermediates, reactive nitrogen intermediates, and other antimicrobial peptides infected macrophages can be activated to kill or limit replication of *Salmonella* [114,115]. Other massive inflammatory response, characterized by recruitment of innate immune cells, such as neutrophils, DCs, inflammatory monocytes and macrophages are also induced upon *Salmonella* invasion [116,117]. Neutrophils have been reported to prevent dissemination of the bacteria [118] and inflammatory monocytes are important source of anti-microbial factors [117]. Macrophages can also sense bacterial flagellin by NLRC4 to produce proinflammatory IL-18 via activation of caspase-1 [119,120]. DCs can present *Salmonella* antigen to naïve CD4 T cells to activate adaptive immune responses.

Host adaptive immune responses play a significant role to prevent *Salmonella* infection. Different studies have shown the presence of *Salmonella*-specific early CD4 T cell responses after oral infection using T-cell receptor transgenic mice [121,122]. *Salmonella*-specific CD4 T-cell activation occurs earliest around 3-6h post infection within Peyer's patches. The effector functions of distinct proinflammatory cytokine production by CD4 T cells during *Salmonella* infection is mainly acquisition of Th1 effector abilities via enhanced secretion of IFN-γ, TNF-α and IL-2 [123,124]. Appropriate activation of Th1 cells continue to expand and around 2-3 weeks after infection eventually comprise ~50% of total CD4 T cells in spleen [124]. However, very little evidence suggests contribution of Th1 effector cells for bacterial clearance at early stage of infection. A recent study demonstrated alterations in the potency of regulatory T cells during *Salmonella* infection reduces Th1 activity and increases

bacterial growth [125]. Th17-associated cytokines IL-17 and IL-22 play a major role during *Salmonella* infections. Accumulation of these cytokines occurs rapidly after oral infection with *Salmonella* [126,127]. Increased bacterial loads in systemic tissues have also been observed in IL-17 deficient mice compared to wild-type mice [128]. IL-23-dependent production of IL-22 was later found to contribute in-vivo bacterial clearance [129]. A probable contribution of Th17 cells might be via neutrophil infiltration to intestinal tissues, since IL-17 deficient mice showed defective neutrophil recruitment abilities [130]. Together, these studies indicate a strong involvement of Th17 cells and associated cytokines to provide protection against *Salmonella*-induced infections.

Contribution of microbiota to *Salmonella* infection:

The microbiota provides protection against enteric infection specifically in *Salmonella* induced gastroenteritis via different mechanisms (Figure 3B). Early studies have demonstrated that germ-free mice are more susceptible to infection than conventionally raised mice [131,132]. It has been shown that *Salmonella enterica* serovar Typhimurium is capable of colonizing mice harboring low complexity microbiota, but is rapidly eradicated after co-housing with conventionally raised mice [133]. The gut microbiota produces distinct metabolites such as short chain fatty acids (SCFAs). Butyrate down-regulates virulence genes, i.e. type III secretion system of *Salmonella enterica* and decreases its ability to invade or induce apoptosis of host cells *in vitro* [134]. It also has been demonstrated that SCFAs and other organic acid such as lactate can reduce pH locally to a level below the optimum for *Salmonella* to grow [135,136]. Moreover, several studies have revealed that commensal bacteria-driven MYD88-dependent and NOD2-dependent signals provoke intestinal paneth cell expression of different antimicrobial peptides, i.e. cryptdins, which limit *S.* Typhimurium penetration of epithelial barrier [137–139]. Some members of the family Porphyromonadaceae have shown to protect *S.* Typhimurium-induced colitis via unknown mechanism, but do not provide direct colonization resistance [140]. *Bifidobacterium infantis* has shown to increase the number of Tregs that reduced disease severity of *S.* Typhimurium infection [141]. A recent study has revealed protection against

Salmonella via IL-18 mediated induction of Th1 and Th17 cells provided by gut commensal protozoa, *Tritrichomonas musculis* [142]. Another recent study demonstrated induction of systemic IgG by groups of selective gut symbiotic gram-negative bacteria and elevated systemic IgG conferred protection against systemic *Salmonella* infection by directly coating bacteria to promote killing by phagocytes [143]. These studies together strongly support that the intestinal microbiota play significant role to protect host against enteropathogen infections.

1.3 Alteration in microbiota composition affects host pathophysiology

The intestinal microbiota has constantly been exposed to different dietary and environmental pressure. While a stabilized and healthy microbiota composition is indispensible for host health, in recent years many of modern multifactorial diseases have been associated with the alteration in microbiota composition. Such altered composition of the gut microbiota is termed as dysbiosis and this affects both relative abundances of distinct groups of bacteria as well as the functionalities of the microbial community. Accordingly definition of dysbiosis can be explained as functional and compositional alterations of gut microbiota, which is driven by a set of host-related and environmental factors that perturb the microbiota by exceeding its resistance and resilience capabilities [144]. However, among healthy individuals interindividual variability in microbial communities makes dysbiosis difficult to define. Similarly variability in the microbial composition is also present in the laboratory animals due to differences in vivaria and diet that can strongly influence experimental settings [145]. Dysbiosis can be categorized based on its characteristic features such as, bloom of certain pathobionts, loss of commensals, loss of diversity etc. Pathobionts are the otherwise under-represented or potentially harmful bacteria in the GI tract and outgrowth of such pathobionts can enhance infection and inflammation, i.e. Enterobacteriaceae [146]. Conversely, loss of residing beneficial commensals can be functionally important to enhance disease severity and restoration of

abolished bacteria or their functional product can eventually recover disease outcome. As an example, inflammation caused by *Clostridium difficile* has been shown to ameliorate by colonization with commensal *Clostridium scindens* [147]. In addition, decrease in overall diversity of the intestinal bacteria has also been associated in dysbiosis context, i.e. IBD, dietary compostion, AIDS and type I diabetes [148–151].

Dysbiosis can be caused by many different factors that influence microbiota composition. Notably numerous infections and inflammatory conditions have been associated to dybiosis formation. In mouse models, *Citrobacter rodentium* and *Salmonella* Typhimurium infection as well as chemically induced inflammation cause dysbiosis [152,153]. Diet can result in both short-term and long-term effects on microbiota composition. Both low-fibre and high-fat diets have been shown to reduce microbial diversity in mice [149,154]. Host genetics also play a crucial role in shaping intestinal microbiota [155]. Finally transmission across generations and environmental transmission also play major role to reshape microbiota composition.

Dysbiosis, once established, can affect both local and systemic host immunity. Alteration in microbiota composition is sensed by the host resulting in a different immune activation state and promoting disease conditions. For example, *Porphyromonas gingivalis* has been reported to disrupt homeostatic balance of oral commensal microbiota leading to inflammatory disease development [156]. *P. gingivalis* can promote MYD88 degradation, followed by inhibition of antimicrobial responses [157]. Dysbiotic microbiota modulated metabolites, such as taurine, histamine, spermine have been shown to influence NLRP6 signaling activity and modulate host ability to produce antimicrobial peptides [158]. A similar study showed that transfer of dysbiotic community from IL-22 deficient mice reduces expression of IL-22-induced antimicrobial protein in wild type mice [159]. Dysbiotic communities affecting host adaptive immunity have also been reported. *Sutterella* spp. are associated with low sIgA secretion and transfer of these microbiota to healthy host also confers low sIgA secretion and enhanced susceptibility to inflammation [160]. Although numerous studies suggested strong influence of dysbiotic community to reshape host immune system, however it is still

unclear, whether dysbiosis can directly cause disease manifestation in healthy host.

1.4 Mouse models and variables affecting host-microbiota interaction study

Mouse models are crucial laboratory experimental systems in biomedical research to study microbial communities in mammalian host. Although significant differences exist between mice and humans in terms of anatomy, physiological responses and microbial composition, mice still represent a well-characterized model to investigate universal responses. Maintenance under standardized conditions and manipulation of microbiota at will gives a unique opportunity to study how the microbiota affects host pathophysiology. Several different mouse models played major role in the field of microbiota study. Germ free (GF) mice completely lacking the microbiome have been used to study effect of different groups of microbiota in host immune system by monoassociation, conventionalization or transfer of distinct bacterial groups to GF mice [161]. Isobiotic mouse lines exposed to only a defined set of bacteria are also attractive experimental system to analyze microbiome effects [162]. The idea of using standardized minimal microbiota date back to beginning of mid 1960's when efforts were taken to reduce effects of different bacterial communities across experimental populations and still now "altered Schaedler flora" (ASF), a community of eight bacterial members is being widely used [163–165]. Another important tool for microbiota study is a humanized animal model. It has been demonstrated that mice humanized with different human donors result a similar microbiome composition and metabolic profile with preservation of individual-specific features [166]. Many recent projects have focused to design minimal functional human microbial consortia that are easy to manipulate, maintain and interpret [167,168].

Studies performed in mouse models to investigate different phenotypes specially influence of the gut microbiota on host often demonstrate lack of reproducibility. Several variables may play role in such discrepancies on observed phenotypes. One important variable is mouse strain background.

Strain background contributes to many experimental phenotypes and also play a role in composition of microbiome [169]. Many cases age dependent experimental phenotypes can also be observed [170]. Other host factors, such as, gender, vertical transmission from parents can also affect microbiota-mediated phenotypes. Several environmental factors affect significantly experimental phenotype, i.e. housing conditions, diet, handling of animal etc. Comparison for an experimental phenotype between mouse lines obtained from different housing conditions may significantly affect the result. Studies demonstrated that conventionally raised mice often harbor pathobionts like Helicobacteraceae, murine norovirus or protozoa etc. that can affect experimental phenotype. Indeed *IL-10*$^{-/-}$ mice have shown to develop spontaneous colitis only under conventional housing conditions [171]. Although many commercial vendors and academic institutions maintain pathogen free mouse facilities still many of them vary greatly among their microbiota composition [172] and in many cases harbor potential pathobionts like SFB, Prevotellaceae etc. Moreover, factors like lighting, temperature, bedding material also affect microbiota composition and mouse phenotypes. Food and water treatment and sterilization have shown to alter microbiome and very little is known about effect of different brands of standard animal feed that eventually can affect microbiota and microbiota-mediated phenotypes.

Several attempts have taken to obtain proper solutions to deal with variables affecting microbiota and experimental phenotypes. One of the best solutions has been considered as to use littermate controls [145]. Fecal transplantation or co-housing to equalize microbiota and its effect has also been demonstrated to transfer experimental phenotypes. Re-derivation of different gene-deficient mouse lines used in a same study by embryo transfer into a similar microbiota condition can also be considered to reduce some variability effects.

1.5 Targeting microbiota as therapeutic intervention

Host and microbiota acts in concert to maintain health state of an individual. Gut microbiota and microbiota modulated host pathways have long been considered to target as therapeutic intervention for diseases not only localized

in the intestine, but also involving systemic sites. Microbiota manipulation as a therapy has been carried out by use of probiotics, prebiotics, synbiotics, antibiotics and fecal microbiota transplantation (FMT). Many studies have demonstrated use of probiotics, live microoganisms, may confer health benefit. Most commonly used probiotics include *Lactobacillus* spp., *Bifidobacterium* spp., *E.coli*, *Streptococcus* spp. and some *Enterococcus* spp [173]. Mechanisms by which probiotics provide benefits involves, enhancement of GI barrier function, modulation of local and systemic immune responses, antagonizing pathogenic bacteria, production of beneficial metabolites etc. Providing selective ingredients known as prebiotics to change microbiota composition is another way to enhance host health. Examples of prebiotics are dietary fiber, non-starch polysaccharide and some oligosaccharides. A mixture of probiotics and prebiotics, commonly known as synbiotics, has also been applied to beneficially affect host. Common combinations include *Bifidobacteria* and fructooligosaccharides, *Lactobacillus rhamnosus* GG and inulin etc. Targeting microbiota by antibiotic has considered as therapeutic option. However, side effects of superinfection have discouraged use of antibiotic to manipulate microbiota. FMT consisting of infusion of fecal suspension from a healthy individual to gut of another one has shown to have better durable alteration of recipient's gut microbiota compared to probiotics [174]. Suggested mechanisms for FMT include competition for nutrients, direct inhibition of pathogen growth and host immune modulation.

Many clinical trial studies have been undertaken to observe impact of microbiota manipulation under disease conditions. Compared to Chron's disease, microbiota manipulation has shown promising effect against ulcerative colitis. The strongest evidence derived from clinical trials conducted with VSL#3, a probiotic mixture containing 900 billion viable lyophilized bacteria represented by four strains of Lactobacilli, three of Bifidobacteria and one of *Streptococcus salivaris* [175,176]. Kruis et al. reported equivalent percentages of efficacy and safety in maintaining remission in 162 UC patients giving probiotic *E. coli* Nissle 1917 compared to 165 patients receiving mesalazine [177]. Successful efficacy of probiotics have also been reported against *Clostridium difficile* associated and other antibiotic-

associated diarrhea [178]. However, in the last years, FMT has been recognized as an promising treatment for recurrent *C. difficile* infection by remodulation of intestinal microbiota [179].

As many studies demonstrate targeting microbiota can be a useful therapeutic intervention in many disease conditions, as valuable it becomes to understand the detailed mechanisms how microbiota and its effects to the host can be utilized to generate improved therapeutic strategies. Due to significant individual variations among human and their microbiota, more and more studies are also emphasizing to find out microbiotal biomarkers to enhance personalized therapies.

1.6 Aims of this study

An individual's composition of the microbial communities in the GI tract reflects the dynamic development of host and microbes to ideally achieve a homeostatic, mutually beneficial state. But alterations in this homeostatic balance have been associated to many local and systemic disorders. However, it still remains to be elucidated whether altered gut microbiota or specific members of the intestinal commensal can directly influence host pathophysiology. Therefore the main focus of my PhD thesis is to investigate how distinct commensal bacterial communities or specific members of the microbiota can interact with different arms of the host immune system to directly influence the susceptibility to different inflammatory diseases in the GI tract.

Studies investigating the effect of the microbiota in mouse model rely on specific tools, such as standardized housing conditions, defined disease models and mouse strains with identical genetic backgrounds. We have devised strategies to control such variables to investigate the aims of this study:

1. The first aim of my thesis is to understand whether distinct microbial communities in the GI tract can directly influence disease susceptibility and whether different microbial communities trigger comparable intestinal pathologies via shared or distinct pathways. Here we applied

a mouse model of human ulcerative colitis using dextran sulfate sodium (DSS). To investigate the influence of distinct microbial communities we monitored susceptibility of DSS colitis to wild type mice obtained from different commercial vendors with similar genetic background and only differing in their microbiota composition. Furthermore different gene-deficient mouse lines rederived into a defined isobiotic microbiota that can easily be manipulated by cohousing will allow us to thoroughly monitor involvement of host immune system to mediate microbiota-induced colitis. We have also employed advanced techniques for characterizing distinct microbial communities and immunophenotyping of host harboring different microbiota.

2. The second aim of my thesis is to evaluate whether specific member of the intestinal microbiota can modulate host immunity to influence the outcome of GI inflammatory disease. Here we studied, SFB, a previously reported intestinal microbial member having immunomodulatory effect. Studies demonstrated SFB could enhance different immune-mediated diseases by modulating host immune system. In this study we focused whether SFB through its *in vivo* host immunomodulatory effects can provide any benefit to the host during enterpathogen infection. We have used streptomycin mouse model of *Salmonella* Typhimurium induced gastroenteritis. Use of specific tool for cytokine and transcription factor knock-in reporter mice rederived into a defined isobiotic microbiota allowed us to monitor effect of SFB directly *in vivo* to modulate host immune cells producing different cytokines. Using such tool we could also characterize gene-signatures expressed in SFB imprinted immune cells.

Together these investigations should enrich our knowledge about direct influence of gut microbiotal communities or specific member on modulating host immunity during homeostasis and inflammation.

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CHAPTER 2

Distinct microbial communities trigger colitis development upon damage to the intestinal barrier via innate or adaptive immune cells

2.1 Summary

Inflammatory bowel disease is a group of heterogeneous diseases characterized by chronic and relapsing mucosal inflammation. Alterations in microbiota composition have been proposed to contribute to disease development, but no uniform signatures have yet been identified. Here, we compare the ability of a diverse set of microbial communities to exacerbate intestinal inflammation after chemical damage to the intestinal barrier. Strikingly, genetically identical wild type mice differing only in their microbiota composition varied strongly in their colitis susceptibility. Transfer of distinct colitogenic communities in gene-deficient mice revealed that they triggered disease via opposing pathways either independent or dependent on adaptive immunity, specifically requiring antigen-specific CD4+ T cells. Our data provide evidence for the concept that microbial communities may alter disease susceptibility via different immune pathways despite eventually resulting in similar host pathology. This suggests a potential benefit for personalizing IBD therapies according to patient-specific microbiotal signatures.

2.2 Introduction

Inflammatory bowel disease (IBD) is a complex group of incurable inflammatory disorders comprising of Crohn's disease (CD) and Ulcerative colitis (UC). These diseases develop in different areas of the gastrointestinal (GI) tract and are also characterized by different types of inflammatory responses [1,2]. Although the etiopathogenesis of IBD development is not fully

understood, numerous studies support the hypothesis of IBD as a pathological immune response against microbial and environmental antigens in genetically predisposed individuals. The relative contribution of innate and adaptive immune cells and various cytokines to the development of IBD has been controversially debated [3]. Nonetheless, an imbalanced interaction between the host immune system and gut microbiota is thought to play a pivotal role in disease manifestation and maintenance [4–6].

In healthy individuals, microbiota and host maintain a symbiotic relationship, in which the microbiota provides benefits to the host by contributing to resistance against pathogens, to metabolic processes and to the proper development of the immune system [7]. Notably, the composition of the microbiota can vary greatly among individuals being influenced by genetic predisposition and environmental factors, e.g. diet and use of antibiotics and other drugs [8,9]. Numerous human disease conditions have been associated with imbalances in the composition of the gut microbiota, so-called dysbiosis, however, whether these changes contribute directly to the development of the disease or reflect an altered physiology of the host remains debated in many instances [7,10,11]. Mouse models of human diseases have been vital to establish a causal role for the microbiota in the development of metabolic syndrome, behavioral abnormalities and infections. Moreover, in various mouse models of IBD the microbiota and in some cases specific members have been shown to influence disease outcome [12]. Examples for IBD mouse models that lack disease development in the absence of any microbiota are the *Il10*$^{-/-}$ model of colitis and the *TNF*^{deltaARE} model of ileitis [13,14]. Furthermore, disease development in these models is impaired or delayed in specific-pathogen free (SPF) conditions compared to conventional housing conditions demonstrating that particular microbiota members or distinct communities only present in conventionally housed mice modulate disease onset. Specifically, Enterobacteriaceae in *Tbet*$^{-/-}$*Rag2*$^{-/-}$ mice [15] as well as *Bacteroides spp*. [16], *Helicobacter spp*. [17] and *Bilophila wadsworthia* [18] in *Il10*$^{-/-}$ have been shown to enhance intestinal inflammation.

The acute dextran sulfate sodium (DSS) colitis model of human UC is considered to be largely dependent on innate immunity [19]. We previously

demonstrated that the dysbiotic microbiota of Nlrp6 inflammasome deficient mice has the ability to directly enhance DSS colitis severity, yet, the effector mechanism remained unknown [20]. Notably, a recent study identified that specific metabolites of this dysbiotic community actively modulate innate immune signaling and subsequently the host-microbiota interface [21]. Subsequently, similar dysbiotic communities with the ability to modulate the severity of DSS colitis have been described in other gene-deficient mice [22–24]. However, it remains to be examined whether different colitogenic communities trigger intestinal pathologies via shared or distinct immune pathways. This knowledge could potentially explain the variable roles that have been suggested for various immune effectors and pathways for IBD pathogenesis.

In the present study we have characterized the susceptibility of mouse lines differing only in their microbiota composition towards DSS colitis. Besides the dysbiotic community (DysM) from *Nlrp6*$^{-/-}$ mice, interestingly also certain but not all SPF communities demonstrated the abilities to cause severe intestinal inflammation in immunocompetent mice. While the colitogenic communities displayed differences in the relative abundance of specific bacterial families, they all shared similarly decreased ratio of Firmicutes to Bacteroides compared to communities causing only mild disease. Strikingly, mice displayed different inflammatory responses depending on their intestinal microbiota composition, either characterized by infiltration of neutrophils or the presence of proinflammatory CD4+ T cells. By utilizing gene-deficient mice and antibody-mediated depletion of T cell subsets we demonstrated that the DysM but not another colitogenic community depends on CD4+ T cells to exacerbate DSS colitis severity. Our data identifies that specific interactions between colitogenic communities and host immune pathways drive colitis development via distinct mechanisms. This highlights the potential to stratify patients by screening the microbiota and to develop microbiota-centered interventions for personalized IBD therapy.

2.3 Experimental procedures

Mice: Wild type and all transgenic mice, *Rag2*$^{-/-}$, *Tcrbd*$^{-/-}$, *muMT*$^{-/-}$, *Tcrd*$^{-/-}$, OTII, *CD4*$^{-/-}$, *CD8*$^{-/-}$, IL-17A^{GFP} IFN-γ^{Katushka} FoxP3^{RFP} reporter mice used in the study were on C57BL/6N background and have been rederived into SPF-1 microbiota by embryo transfer and bred at the specific pathogen free animal facilities of Helmholtz Centre for Infection Research (HZI). *Nlrp6*$^{-/-}$ mice were obtained from Yale University, USA and subsequently bred under conventional housing conditions at the HZI. Other donor microbiota for different composition were purchased from different commercial vendors (Janvier, Taconic, Harlan). Germ-free C57BL/6NTac mice were bred in isolators (Getinge) in the germ free facility of the HZI. All experiments were performed with 10-14 weeks old age-matched and gender-matched animals. Both male and female animals were used for every experiment to exclude influence of gender.

Microbiota manipulation: Alteration of microbiota composition was conducted by cohousing or fecal transplantation. For cohousing both recipient and donor mice were cohoused at least 4 weeks prior starting experiment. For fecal transplantation donor mice were euthanized, intestinal content was collected in BBL thioglycollate media (BD Bioscience) and homogenized by vortexing. To remove course particle under anaerobic conditions the content was filtered through 70um sterile filter. After centrifugation (10min, 500g, 4^0C), the pellet containing fecal bacteria was resuspended in BHI medium (Sigma-Alrich). After 2-hr of starving recipient mice were orally gavaged with a total 200ul of fecal bacterial content. Again a 4 week time period was given for a successful establishment of fecal transplanted bacteria (unless mentioned otherwise). Every microbiota manipulation was further confirmed by 16S rRNA sequencing of fecal bacteria.

DNA isolation and 16S rRNA microbial community analysis: Fresh stool samples of mice were collected and immediately stored at -20°C. DNA was extracted according to established protocols using a method combining

mechanical disruption (bead-beating) and phenol/chloroform-based purification [25]. Briefly, sample was suspended in a solution containing 500µl of extraction buffer (200 mM Tris, 20mM EDTA, 200mM NaCl, pH 8.0), 200µl of 20% SDS, 500µl of phenol:chloroform:isoamyl alcohol (24:24:1) and 100µl of 0.1 mm zirconia/silica. Samples were homogenized twice with a bead beater (BioSpec) for 2 min. After precipitation of DNA, crude DNA extracts were resuspended in TE Buffer with 100µg/ml RNase and column purified to remove PCR inhibitors (BioBasic). Amplification of the V4 region (F515/R806) of the 16S rRNA gene was performed according to previously described protocols [26]. Samples were sequenced on an Illuminia MiSeq platform (PE250). Filtering of sequences for low quality reads (q>=30) and barcode-based binning was performed by using QIIME v1.8.0 (Caporaso et al., 2010). Reads were clustered into 97% ID OTUs using UCLUST reference OTU picking, followed by taxonomic classification using the RDP Classifier executed at 80% bootstrap confidence cut off [27,28]. Sequences without matching reference dataset, were grouped as *de novo* using UCLUST. The OTU absolute abundance table and mapping file were used for statistical analyses and data visualization in the R statistical programming environment package phyloseq [29]. To determine bacterial OTUs that explained differences between microbiota settings, linear discriminant analysis (LDA) effect size (LEfSe) method were used [30]. OTUs with Kruskal-Wallis test <0.05 and LDA scores >3.5 were considered informative.

Dextran sodium sulfate (DSS) induced colitis: To induce acute colitis, mice were provided 2% (w/v) DSS (molecular mass= 36-50 kDa, MP Biomedicals) in drinking water for 7 days followed by 7 days of access to regular drinking water. Daily clinical assessment of DSS-treated animals included body weight loss measurement, stool consistency, detection of blood in stool. Experimental samples were collected at day 0, 5, 7 of DSS treatment.

Histology: At day 5 of DSS-treatment colon samples were collected, rolled up to “swiss roles”, fixed in 4% neutrally buffered formaldehyde and embedded in paraffin according to standard histological procedures. Sections of 3µm

thickness were stained with hematoxylin-eosin (HE) and evaluated by light microscopy blinded to the experimental groups.

The histological scoring used to evaluate the severity of colitis in DSS treated mice microscopically, was adapted from the TJL-score, which was developed for scoring colitis in mice by The Jackson Laboratory [31]. The alteration of the score has been previously described [32]. The colon was divided into a proximal (oral), middle and distal (aboral) section, each of about the same size. The three sections were scored for the general criteria: severity (0-3), ulceration (0-3), oedema (0-3), goblet cell metaplasia (0-3), and area involved (0-3) where score 0 depicted no alteration to score 3 massive alteration in the given parameters.

The scores were added up to a total of up to 15 per section and the scores of the three sections to a total of up to 45 per colon sample.

Colonoscopy: Colonoscopy was performed using a high-resolution mouse video endoscopic system ('Coloview', Carl Storz, Tuttlingen, Germany). The severity of colitis was blindly scored using MEICS (Murine Endoscopic Index of Colitis Severity), which is based on five parameters: granularity of mucosal surface (0-3); vascular pattern (0-3); translucency of the colon mucosa (0-3); visible fibrin (0-3); and stool consistency (0-3) [33].

Isolation of colonic lamina propria leukocytes (cLPL) and flow cytometry: To isolate cLPL, density gradient centrifugation using Percoll was done as previously described [34]. In brief, colons were collected during steady state and at d5 of DSS treatment. Fecal content was removed, tissues were opened longitudinally, washed with PBS and then shaken in HBSS containing 2 mM EDTA for 20 min at 37°C. Tissues were cut into small pieces and incubated with digestion solution (DMEM containing 1% fetal bovine serum (FBS), 0.25 mg/ml collagenase D, 0.5 U/ml dispase and 5 μg/ml DNase I) in a shaker for 20 min at 37°C. Digested tissues were filtered through 70uM cell strainer (Falcon) and DMEM + 5% FBS was added to inactivate enzymes. The last two steps were repeated until all tissue was digested. After centrifugation, cells were resuspended in 4 ml of 40% Percoll (GE Healthcare)

and overlaid on 4 ml of 80% Percoll. Percoll gradient separation was performed by centrifugation at 450 g for 25 min at 25°C. Cells in the interphase were collected and used as LPL. The collected cells were then suspended in staining buffer containing PBS, 1% FBS and 2 mM EDTA. The following antibodies were used: anti-CD45 (30-F11), anti-CD3 (17A2), anti-B220 (RA3-6B2), anti-CD4 (RM4-5, GK1.5), anti-CD8a (53-6.7), anti-TCR-gamma/delta (GL3), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-MHC class II (M5/114.15.2), anti-CD11b (M1/70), anti-CD11c (N418), anti-Ly6G (IA8), anti-Ly6C (HK1.4) (Biolegend). To distinguish live dead cells AlexaFluor-350 NHS Ester (Life Technologies) was used. Flow cytometry analysis was performed using a BD LSR (BD Biosciences) and data were analyzed with FlowJo software (TreeStar Inc.).

In-vitro T cell activation of cLPL: For T cell activation freshly isolated cLPL (200,000/well) were cultured in 96-well round-bottom plates in complete culture medium containing soluble, plate-bound, anti-CD3 (1mg/ml) and soluble anti CD28 (5mg/ml) (Biolegend) for 3 days. Supernatant were collected after 3 days for cytokines and chemokines measurement.

Collection of colonic tissue homogenate: Colonic tissue homogenate was collected from euthanized mice during steady state and d7 colitis. Colons were excised into proximal and distal colon. Each part was cut longitudinally and cleaned by washing with autoclaved 1x PBS. Parts of proximal or distal colon were weighed and homogenized mechanically using Mini-Beadbeater-96 (Biospec) in NP-40 lysis buffer containing protease inhibitors (Complete Mini EDTA-free, Roche). Protein extracts were centrifuged (10,000 r.p.m. for 5 min at 4°C) and the supernatants were collected as tissue homogenate samples and stored at -80°C.

Cytokine detection- ELISA, multiplex and Legendplex: Concentration of IL-18 in the tissue homogenates was measured using the following commercial ELISA kits: IL-18 (MBL) according to manufacturer's instruction. Different other cytokines and chemokines were measured by using the

ProcartaPlex Multiplex Immunoassay (eBioscience) and FACS based Legendplex kit (Biolegend) according to the manufacturer's instructions.

RNA isolation and quantitative PCR: Tissues were preserved in RNAlater solution (Ambion) and subsequently homogenized in Trizol reagent (Invitrogen). One microgram of total RNA was used to generate cDNA by the protocol for first strand cDNA synthesis using RevertAid RT (Thermo Scientific). RealTime-PCR was performed using gene-specific primer sets (Applied Biosystems) of *Cd4* primer (F: 5'-TAGCAACTCTAAGGTCTCTAAC and R: 5'-GATAGCTGTGCTCTGAAAAC) and Kapa Sybr Fast qPCR kit (Kapa Biosystems) on a LightCycler 480 instrument (Roche). PCR conditions were 95^0C for 60 s, followed by 40 cycles of 95^0C for 3 s and 60^0C for 30 s. Data were analyzed using the the deltaCt method with *hprt* (F: CTGGTGAAAAGGACCTCTCG and R: TGAAGTACTCATTATAGTCAAGGGCA) serving as the reference housekeeping gene.

RNA-Seq Analysis: Total RNA isolation from distal colonic tissue was performed as described at the RNA isolation section. RNA integrity was measured in a Bioanalyzer (Agilent Technologies, USA) and samples were selected according to RNA Integrity Number (RIN) > 9. Isolation of mRNA was performed with Dynabeads mRNA DIRECT Micro Kit (Ambion, USA) using 1ug of total RNA. Furthermore, cDNA synthesis, fragmentation and sequencing library preparation were done using ScriptSeq v2 RNA-Seq Kit (PCR 15 cycles) (Illumina, USA). Sequencing was performed through Illumia Hi-seq 2000 platform in single end mode for 50bp.

We obtained and average of 52,2Mio of reads per sample (n=16). Reads were quality filtered using Trimmomatic with as follow parameters (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:35 HEADCROP:3). After quality control reads were aligned to the mouse reference genome (mm10) using STAR (https://www.ncbi.nlm.nih.gov/pubmed/23104886). Reads count to each gene

was evaluated using HTseq(https://www.ncbi.nlm.nih.gov/pubmed/25260700). Normalization and differential expression were quantified using the DEseq2 package (https://www.ncbi.nlm.nih.gov/pubmed/?term=Moderated+estimation+of+fold+change+and+dispersion+for+RNA-seq+data+with+DESeq2). Differential expressed gene networks were analyzed with Consensus Path DB-mouse webserver (https://www.ncbi.nlm.nih.gov/pubmed/?term=The+ConsensusPathDB+interaction+database%3A+2013+update). Data was visualized using ggplot2 R library.

Antibody-mediated depletion: Anti-CD4 (clone GK1.5) and anti-CD8a (2.43) neutralizing antibodies and an isotype control mAb (clone LTF-2) (Bio X Cell) were used. For each antibody, 200ug injections were given intraperitoneally (i.p.) at day -1, day 3 and day 7 of DSS treatment. Isolating cLPL and splenic lymphocytes followed by flow cytometry assessed depletion efficiency.

CD45Rbhi colitis: CD4+Foxp3-CD45RB(high) cells were transferred adoptively into *Rag2*$^{-/-}$ mice according to the protocol described in [35]. Briefly, splenic lymphocytes were isolated from IL-17A^{GFP} IFN-$\gamma^{Katushka}$ FoxP3^{RFP} triple reporter mice. CD4 enrichment was performed according to the manufacturer instruction using CD4 (L3T4) Microbeads (Miltenyi Biotec). CD4 enriched cells were then stained with CD45RB, CD4. Cells were sorted in BD FACSAria II cell Sorter by gating CD45RB(high), CD4+Foxp3- cells. Anbodies used for staining were anti-CD45RB (C363-16A), anti-CD4 (GK1.5). A total of 500,000 cells were injected i.p./mouse. Disease development was monitored by weighing animals 3 times a week and performing colonoscopy.

Statistical analyses: Statistical analysis was performed using GraphPad Prism program (GraphPad Software). Data are expressed as mean ± SEM. Differences were analyzed by Student's t test and ANOVA. *P* values indicated represent a non-parametric Mann-Whitney U test or Kruskal-Wallis test comparison between groups. *P* values ≤ 0.05 were considered as significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.4 Results

2.4.1 DSS colitis severity is strongly influenced by microbiota composition in SPF mice

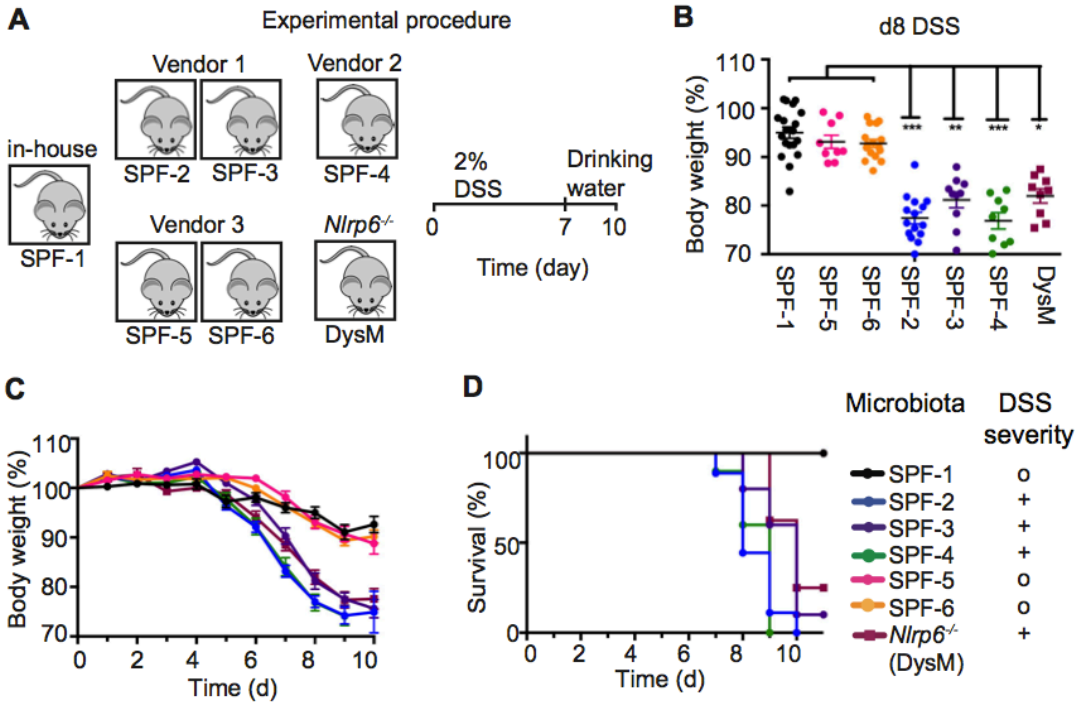


Figure 2.1 Isogenic mice from different breeding facilities demonstrate variable susceptibility to DSS induced colitis.

(A-D) DSS colitis was induced in SPF WT (SPF-1 – SPF-6) and dysbiotic *Nlrp6*$^{-/-}$ (DysM) mice by administering 2% DSS (w/v) for 7 days. Body weight and survival of mice were examined on daily basis for 10 days (A). Body weight on d8 DSS (B), during entire experiment body weight (C) and survival (D) of mice described in Figure 1A. DSS severity is depicted as "o" being mild and "+" being severe. Data represent n=6-18 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test. *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Distinct differences in microbiota composition between isogenic mice from commercial vendors, e.g. the presence of segmented filamentous bacteria (SFB), have been found to influence the outcome of disease models in mice [36]. To investigate whether C57BL/6N mice differ in their susceptibility to intestinal inflammation after chemical-induced damage to the intestinal barrier, we induced DSS colitis in SPF mouse lines obtained from vendors or bred in-

house (Figure 2.1A). After mice were exposed to DSS (2 % w/v) for 7 days severity of disease was compared within lines of SPF mice and to previously described dysbiotic *Nlrp6*$^{-/-}$ mice (Figure 2.1B, 2.1C and 2.1D)[20]. SPF-1, SPF-5, and SPF-6 mice were characterized by a mild colitis with moderate weight loss and no mortality, but SPF-2, SPF-3, and SPF-4 mice as well as dysbiotic *Nlrp6*$^{-/-}$ mice developed a similar severe colitis with profound loss of body mass and mortality (Figure 2.1B, 2.1C and 2.1D).

Next, we investigated fecal microbiota composition before induction of DSS colitis in SPF and DysM mice using 16S rRNA sequencing. Analysis of β diversity using PCoA showed that mice with mild colitis severity (SPF-1, SPF-5 and SPF-6) clustered separately from mice featuring a high severity of colitis (SPF-2, SPF-3, SPF-4, and DysM). We noted a high similarity between SPF-2, SPF-3 (both from different barriers of the same vendor), and SPF-4 mice as well as between SPF-5 and SPF-6 mice (both from different barriers of the same vendor), respectively, whereas SPF-1 and DysM mice clustered distinctly (Figure 2.2A). More detailed analysis revealed that species richness (Chao index) was lower in SPF-1 mice, but that complexity of community structure (Shannon index) was not significantly different between mouse lines (Figure 2.2B). Global changes in the composition of microbiota have been associated with IBD [6], such as decrease in the level of resident Firmicutes and/or Bacteroides and an overabundance of Proteobacteria [37]. We observed a significant expansion of Bacteroides over Firmicutes in colitogenic SPF-2, SPF-3, SPF-4 and DysM mice compared to SPF-1, SPF-5 and SPF-6 mice (Figure 2.2C). Overgrowth in Proteobacteria was highest in DysM mice, followed by SPF-2, SPF-3, SPF-4 and SPF-5 mice and was mostly absent in SPF-1 and SPF-6 mice (Figure 2.2C). A detailed microbiota composition of different groups used in this study is given in Figure 2.2D.

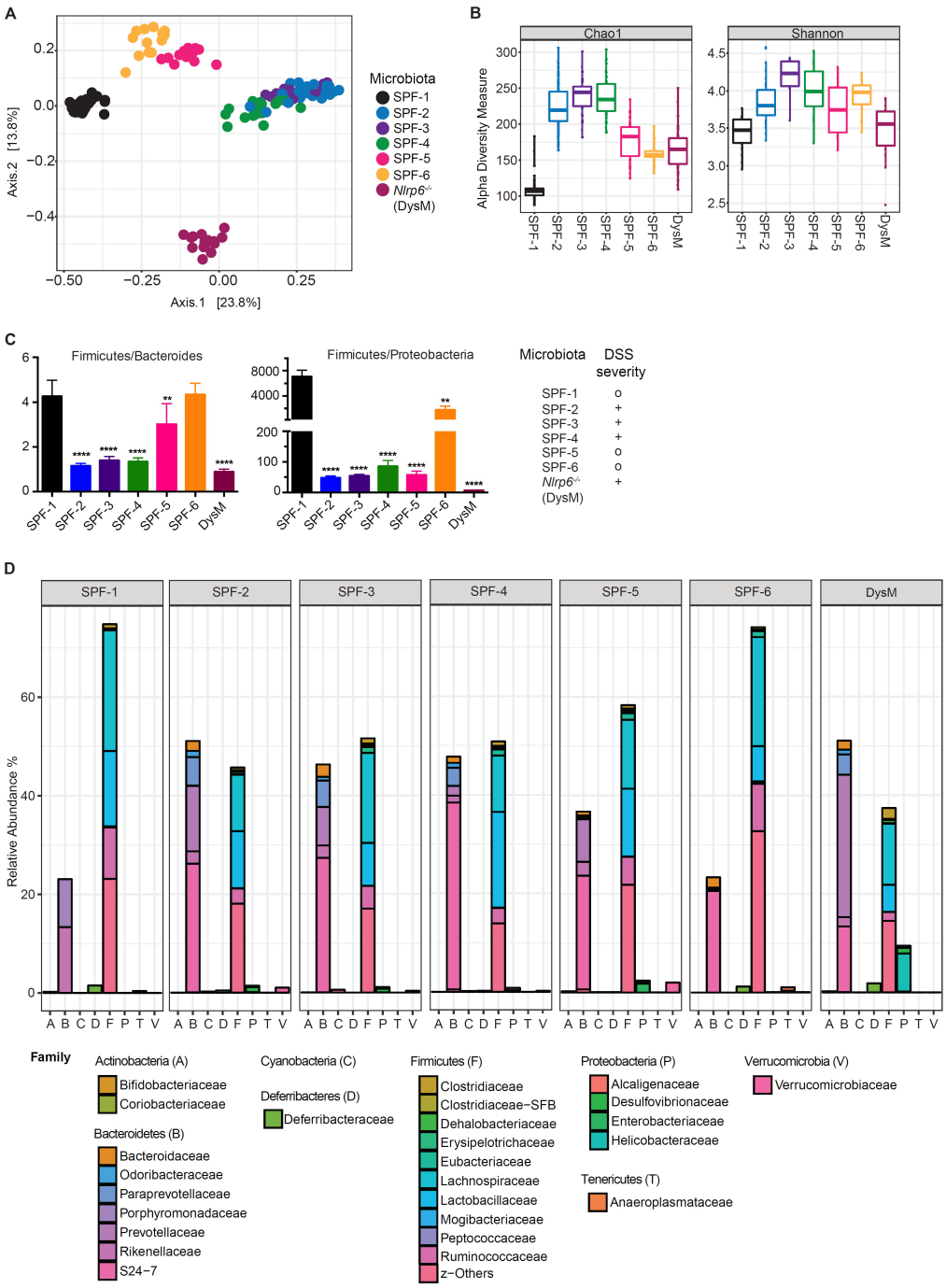


Figure 2.2 Comparison of microbiota composition of different isogenic mouse lines.

(A-D) 16S rRNA sequencing of fecal microbiota of mice described in Figure 1A before DSS colitis induction. Analysis of β-diversity (PCoA) (A), α-diversity (Chao1 and Shannon) (B) ratio of relative abundances between Firmicutes to Bacteroides and Firmicutes to Proteobacteria (C) and relative abundances of different microbial families (D). Data represent n=6-33 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001.

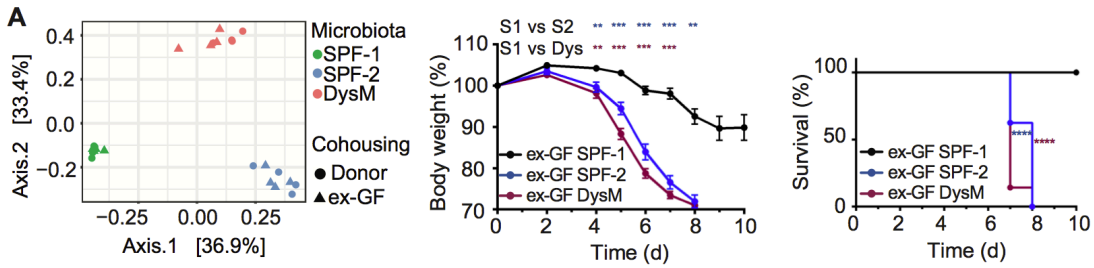


Figure 2.3 Enhanced colitis susceptibility is dependent only on microbiota compositioin.

(A) Germ-free C57BL/6N mice were cohoused with donor SPF WT (SPF-1, SPF-2) and *Nlrp6*$^{-/-}$ (DysM) mice followed by induction of DSS colitis. Analysis of β-diversity (PCoA) before and disease severity (body weight and survival) upon induction of DSS colitis. Data represent n=6-15 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001.

Inbred mouse lines differ vastly in their susceptibility to DSS colitis. To exclude the effect of genetic drift in inbred mice from different sources, we performed cohousing experiments with microbiota donor and germfree recipient mice. We focused on SPF-1 (low susceptibility, higher Firmicutes), SPF-2 (high susceptibility, higher Bacteroides) and DysM mice (high susceptibility, higher Bacteroides and higher Proteobacteria) representing the different colitis outcomes and microbiota compositions. Transfer of the donor microbiota into GF recipient (exGF) mice was confirmed by 16S rRNA sequencing (Figure 2.3A). Upon induction of DSS colitis exGF mice phenocopied the respective donor mice supporting that the differences in colitis severity were dependent on the microbiota (Figure 2.3A). Similar microbiota-driven phenotypes were confirmed for SPF-5 and SPF-6 communities (data not shown). This data demonstrate that distinct types of microbial communities that are stably maintained in WT mice are able to alter the host's susceptibility to DSS colitis.

2.4.2 Transfer of colitogenic microbial communities into an immunocompetent host induces distinct patterns of host gene expression and alters colitis susceptibility

Next, we investigated whether the degree of colitis severity was also transferable between SPF mice with variable DSS colitis susceptibility similar to what has been observed for Nlrp6 inflammasome deficient mice (DysM)[20]. Therefore, we performed cohousing experiments of mice featuring a mild colitis (SPF-1) with mice having a high colitis severity (SPF-2 and DysM). Cohousing for 4 weeks resulted in a reshaping of the microbiota in SPF-1 mice cohoused with SPF-2 mice (SPF-1 + SPF-2) and DysM mice (SPF-1 + DysM) compared to SPF-1 control mice, respectively (Figure 2.4A). Moreover, cohousing also transferred colitis susceptibility as indicated by enhanced weight loss and mortality in SPF-1 + SPF-2 and SPF-1 + DysM mice (Figure 2.4B). Since SPF-1 + SPF-2 and SPF-1 + DysM behaved like SPF-2 and DysM mice, we therefore referred to them in the following as cSPF-2 and cDysM (cohoused SPF-2/DysM), respectively. A similar transfer of colitis severity was also achieved by cohousing of SPF-2 with SPF-6 mice (Figure 2.4F) and after fecal transplantation (FT) from SPF-2 and DysM mice into SPF-1 mice (Data not shown). Increased colitis severity in cSPF-2 and cDysM mice was also illustrated by enhanced colon shortening and corroborated by histological characterization of tissue damage as well as endoscopy (Figure 2.4C, 2.4D, and 2.4E). This data demonstrates that distinct types of microbial communities are able to alter the host's susceptibility to DSS colitis even in already colonized immunocompetent recipients.

Induction of DSS colitis has been shown to alter the composition of the intestinal microbiota 38. To identify whether a shared group of commensals alters their abundance during DSS colitis in SPF-1 mice as well as in cSPF-2 and cDysM mice, we compared their fecal microbial communities before and after induction of DSS colitis (d5). Strikingly, β-diversity analysis (PCoA), as well as analysis of relative abundances of different bacterial families revealed minor differences between the two time points for each community, respectively (Figure 2.5A and 2.5C).

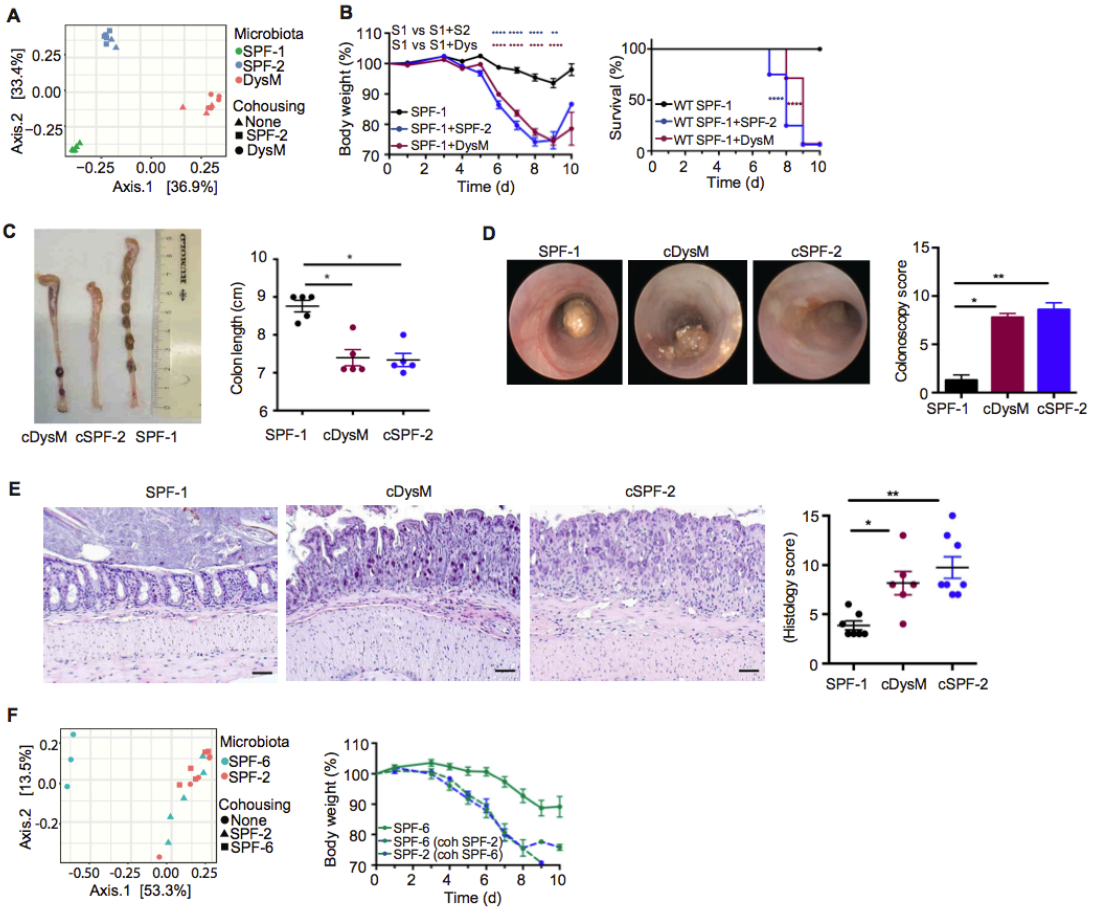


Figure 2.4 Transfer of colitogenic community results in enhanced colitis severity in mice harboring non-colitogenic community.

(A-E) SPF-1 WT mice were cohoused with either SPF-2 WT or DysM *Nlrp6*$^{-/-}$ (SPF-1 + DysM) mice resulting in SPF-1 + SPF-2 and SPF-1 + DysM mice, respectively. Analysis of β-diversity (PCoA) of donor and recipient mice before induction of DSS colitis (A).

(B-E) Acute DSS colitis was induced and body weight and survival of microbiota recipient mice was monitored for 10 days (B). Representative pictures and colitis severity score by colonoscopy performed on day 6 after colitis induction (D). Colon length was measured 5 days after induction of DSS colitis. Representative image of excised colons (C). Histological analysis of distal colon was performed 5 days after induction of DSS colitis (E). Representative pictures of H&E-stained colon sections. Bar represents approx. 50µm.

(F) SPF-6 WT mice were cohoused with SPF-2 WT resulting in SPF-6 (coh SPF-2) and SPF-2 (coh SPF-6) mice. Analysis of β-diversity (PCoA) of non-cohoused SPF-6 and cohoused SPF-6 (coh SPF-2) and SPF-2 (coh SPF-6) mice before induction of DSS colitis. Acute DSS colitis was induced and body weight of mice was monitored for 10 days. Data represent n=4-16 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test (B) and nonparametric Kruskal-Wallis test (C,D,E) *p < 0.05; **p < 0.01; ***p< 0.001; ****p< 0.0001.

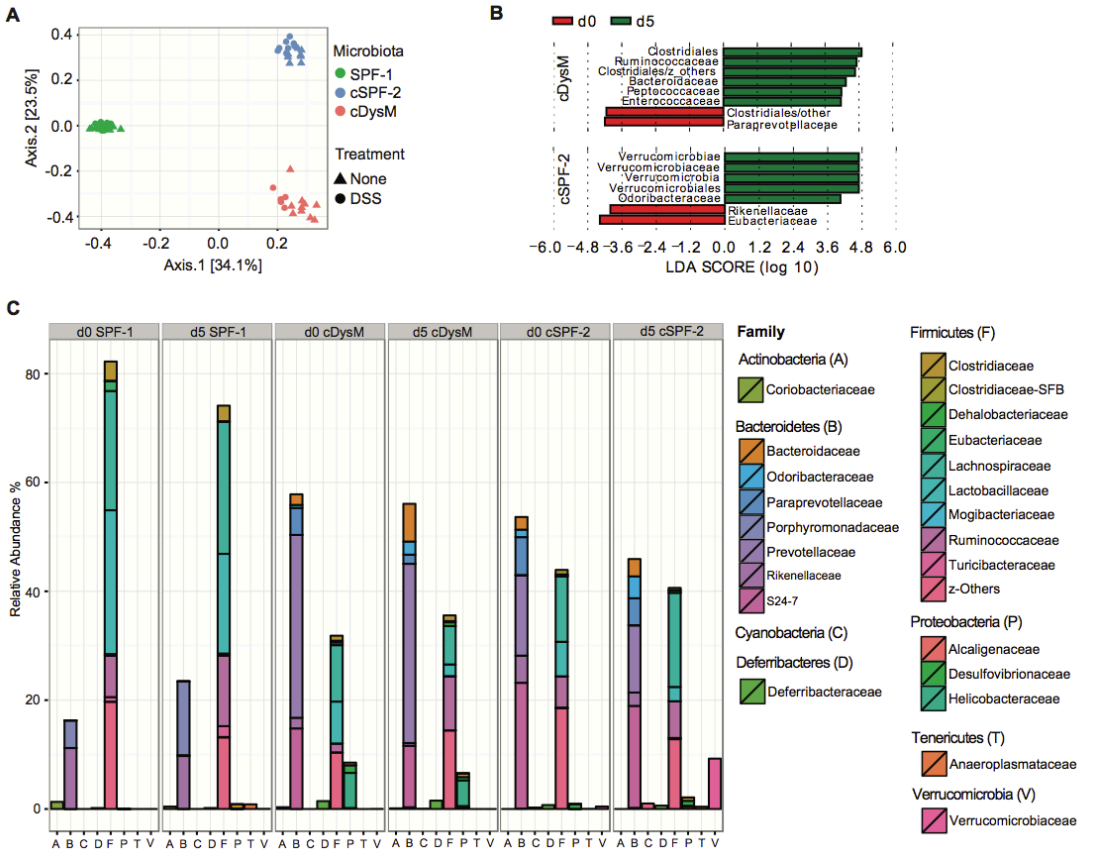


Figure 2.5 Alteration in microbiota composition before and after colitis induction.

(A-C) 16S rRNA sequencing of fecal microbiota from WT SPF-1, cSPF-2 and cDysM at d0 and d5 of DSS colitis. Analysis of β-diversity (PCoA)(A). Analysis of differentially abundant microbial families in cDysM and cSPF-2 mice at d0 and d5 DSS by LEfSe (Kruskal-Wallis test $p<0.05$, LDA 4.0)(B). Relative abundances of different microbial families are displayed (C). Data represent n=5-11 mice/group as mean ± SEM from at least two independent experiments.

Minor alterations included an increase in Verrucomicrobiaceae in cSPF-2 and an increase in abundance of some Bacteroidaceae in cDysM (Figure 2.5B and 2.5C), but no unified changes were observed between the cSPF-2 and cDysM community despite a similar induction of colitis at this time point. Since our data did not show unified alterations in fecal communities after DSS induction, we hypothesized that colitogenic communities already modulate host immunity before disease induction, which in turn results in enhancement of colitis severity.

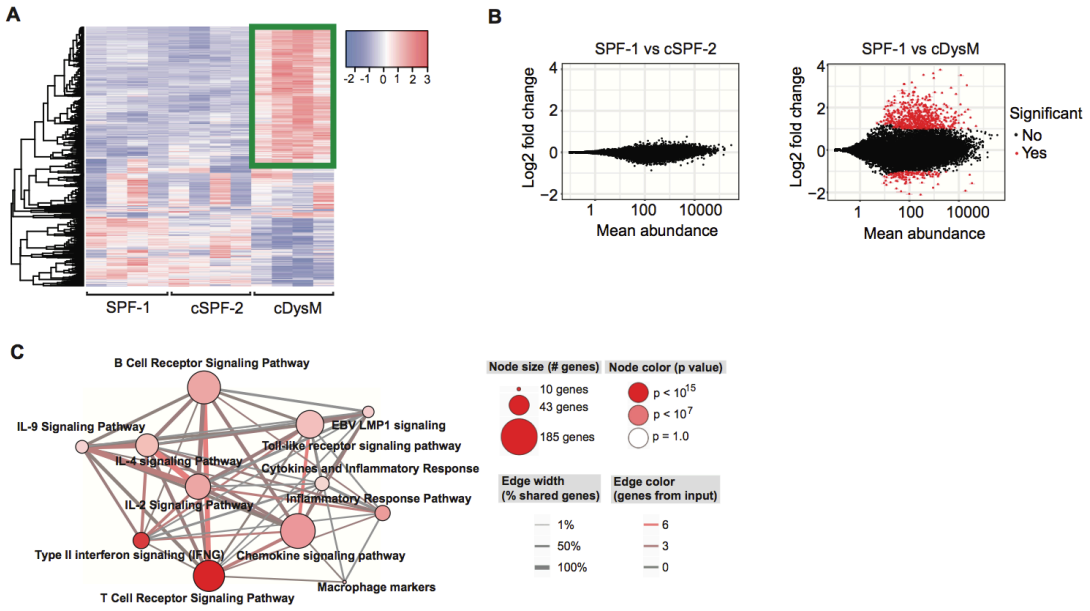


Figure 2.6 Alteration of host responses by colitogenic microbiota.

(A-C) RNAseq analysis from total colonic tissue of WT mice colonized with SPF-1, cSPF-2 or cDysM. Heatmap shows quantification of RNA reads (A). DEseq analysis compares significant up/down-regulation of genes (fold change > 2) in different microbiota conditions (B). Pathway analysis based on GO terms of genes significantly upregulated (2-fold) in cDysM mice compared to SPF-1 (H). Data represent n=4 mice/group.

Hence, global gene expression in colonic tissues of mice harboring either SPF-1, cSPF-2 or cDysM was compared using RNA-seq. Interestingly, SPF-1 and cSPF-2 mice clustered together and separate from cDysM mice (data not shown) with a distinct gene expression signature (Figure 2.6A and 2.6B). Specifically, pathway enrichment analysis showed that many upregulated genes in cDysM were involved in T cell and B cell signaling, as well as cytokine and chemokine signaling (Figure 2.6C). In contrast, despite the fact that a similar colitis severity outcome as observed in cDysM mice, SPF-2 colonization of SPF-1 mice did not result in significant alterations in the host transcriptome (Figure 2.6A and 2.6B). These data together suggest that alteration of the SPF-1 community by colonizing them with the colitogenic SPF-2 or DysM triggers a very different response at the host transcriptional level and that the presence of different microbial families at baseline are enough to enhance such colitogenic microbiota induced pathologies in an immunocompetent host. .

2.4.3 DysM but not SPF-2 microbiota depends on adaptive immune cells to develop colitis

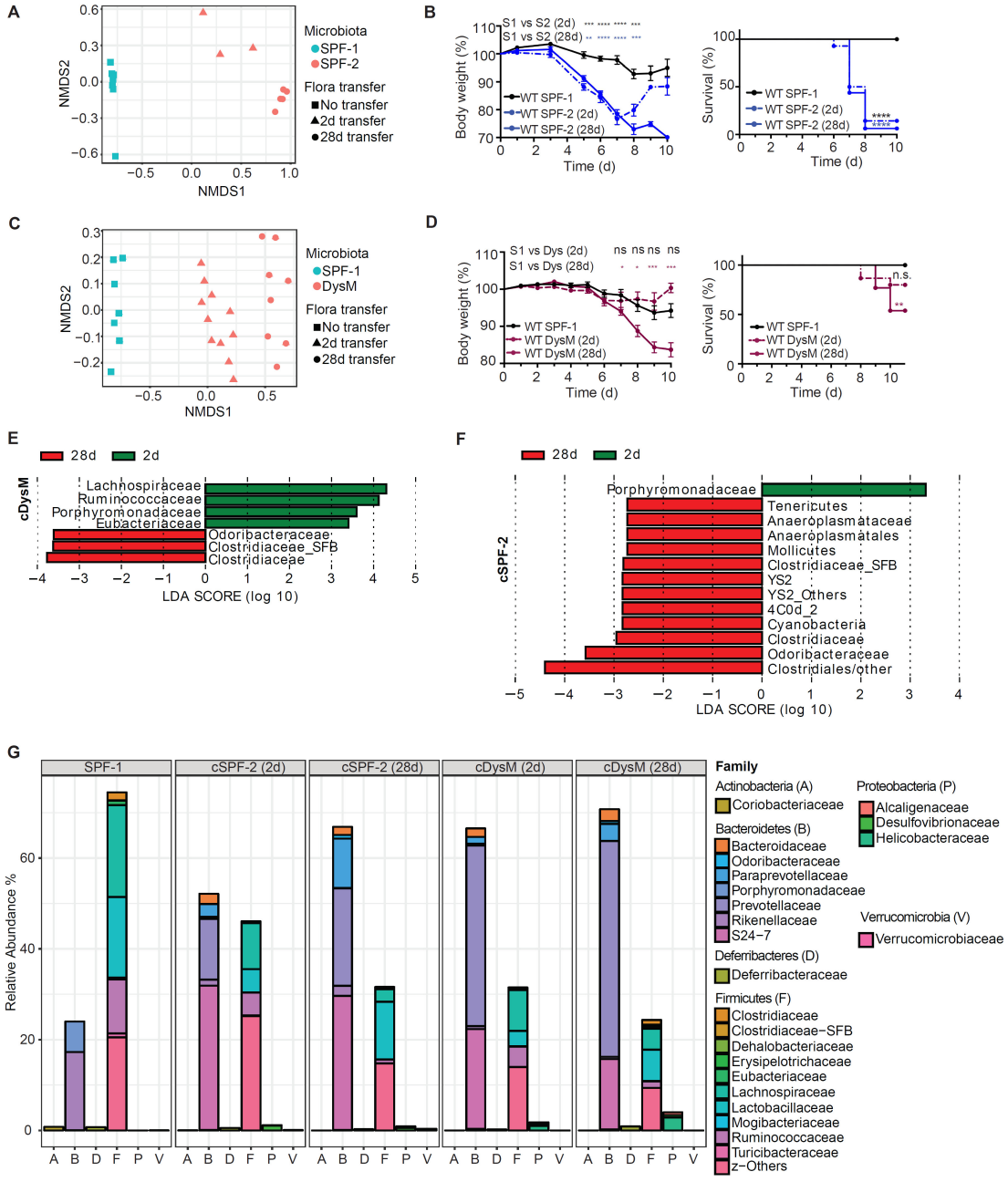


Figure 2.7 Immunomodulation prior DSS induction is required for DysM, but not SPF-2 induced colitis.

(A-G) SPF-1 mice were mock-transferred or received a fecal transplant from *Nlrp6*$^{-/-}$ DysM or WT SPF-2 donor mice 2d or 28d prior to colitis induction.

Since the transfer of the colitogenic SPF-2 community, unlike the DysM community, did not trigger large changes in the host transcriptome in the intestine, we hypothesized that the mere presence of the SPF-2 community may be sufficient to trigger more severe colitis upon damage to the intestinal barrier. Therefore, we assessed disease severity in SPF-1 mice that received FT of the SPF-2 or DysM community 2 days or 28 days prior to disease induction, respectively. Despite minor but detectable differences in communities of mice receiving FT for 2 or 28 days (Figure 2.7A, 2.7C and 2.7G), already a brief colonization with the SPF-2 microbiota was sufficient to transfer exacerbated disease severity that was comparable to the result following extended colonization (Figure 2.7B). In contrast, brief colonization with the DysM microbiota did not transfer heightened disease susceptibility (Figure 2.7D). This inability of the DysM microbiota to transfer colitis severity potentially results from incomplete microbiota transfer, a requirement for extended immunomodulation or priming of adaptive immune responses. Comparison of the communities in mice receiving the DysM FT for 2 or 28 days by LEfSe analysis revealed very minute differences (Figure 2.7E), including a higher abundance of SFB as well as Odoribacteriaceae 28 days after the transfer. Notably, despite successful transfer of colitis severity, communities differed stronger in the case of SPF-2 FT (Figure 2.7F). Interestingly, similar to the DysM FT, SFB and Odoribacteriaceae displayed higher abundances 28 days after SPF-2 transfer. This suggests that these bacteria may not be involved in modulating DSS colitis severity.

Figure 2.7 (cont.) (A, C) PCoA plot of fecal microbiota composition at steady state of WT SPF-1 mice receiving SPF-2 (A) or DysM (C) microbiota for different time periods. (B, D) Body weight and survival of WT SPF-1 mice receiving SPF-2 (B) or DysM (D) microbiota for different time periods during DSS colitis. (E-F) Analysis of differentially abundant microbial families in mice with short (2d) and prolonged (28d) exposure to DysM (E) and SPF-2 (F) were analyzed by LEfSe (Kruskal-Wallis test $p<0.05$, LDA 2.0) before induction of DSS colitis. (G) Relative abundance of different bacterial families in fecal microbiota in mice with short (2d) and prolonged (28d) exposure to DysM and SPF-2 before DSS induction.
Data represent n=3-15 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

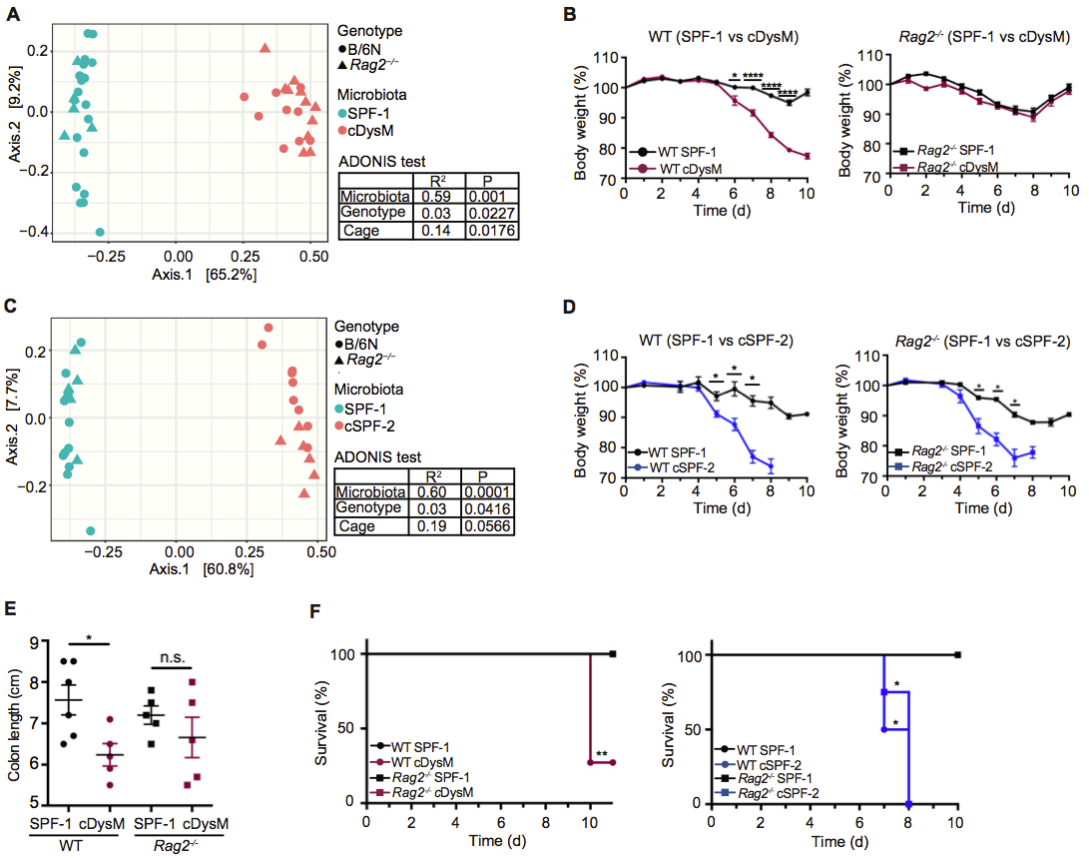


Figure 2.8 Adaptive immune system is important for DysM mediated colitis.

(A-F) SPF-1 WT and SPF-1 *Rag2*$^{-/-}$ recipients were cohoused with donor DysM or SPF-2 and body weight was monitored upon colitis induction (B, D) respectively. Analysis of β-diversity (PCoA) of DysM recipients (A) and SPF-2 recipients (C) is shown along with multivariate analysis of variance (ADONIS test) of variables 'microbiota composition', 'genotype' and 'cage'.

(E-F) SPF-1 and cDysM WT and *Rag2*$^{-/-}$ were sacrificed on d5 of DSS colitis and colons were excised. Colon length was measured (E) and survival of mice with different microbiota are given (F). Data represent n=5-15 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Next, to test whether DysM requires priming of adaptive immunity, we compared the severity of DSS colitis between *Rag2*$^{-/-}$ mice harboring either the SPF-1, cSPF-2 or cDysM communities. Therefore, SPF-1 *Rag2*$^{-/-}$ mice were cohoused with either DysM or SPF-2 donor mice. Strikingly, unlike in WT mice, cDysM could not enhance colitis severity in *Rag2*$^{-/-}$ mice as indicated by similar weight loss (Figure 2.8B) and colon length (Figure 2.8E) between *Rag2*$^{-/-}$ mice with SPF-1 and cDysM. In contrast, cSPF-2 induced severe colitis also in *Rag2*$^{-/-}$ mice as indicated by increased weight loss and mortality (Figure 2.8D and 2.8F). Importantly, we confirmed comparable transfer of the donor communities into WT and *Rag2*$^{-/-}$ mice (Figure 2.8A and 2.8C). We used permutational multivariate analysis of variance (ADONIS) [39], considering the variables “genotype”, “microbiota”, and “cage” to evaluate their relative contribution to variability within the groups (Figure 2.8A and 2.8C). This analysis revealed that genotype contributed only 3% of variability, while microbiota contributed around 60%. Together these data demonstrate that extended immunomodulation and priming of adaptive immunity by DysM but not SPF-2 is required to exacerbate colitis severity.

2.4.4 Colitis development is characterized by the presence of distinct immune signatures in DysM and in SPF-2 mice

Despite a similar disease severity in DysM and SPF-2 mice upon DSS colitis induction, our initial results corroborated the hypothesis that distinct colitogenic communities contribute to disease development via different pathways. To further compare intestinal inflammation induced in cDysM compared to cSPF-2 mice, presence of cytokines and chemokines were measured in tissue homogenates at day 7 of DSS colitis.

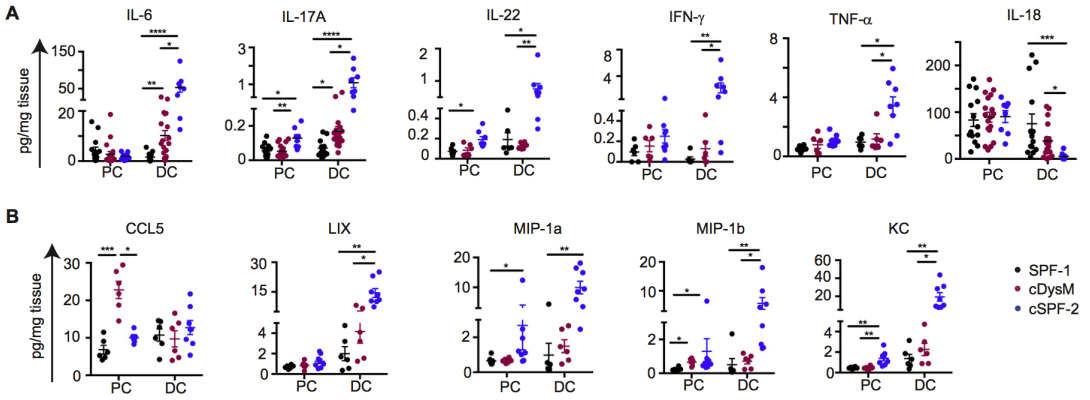


Figure 2.9 Colitis driven by DysM and SPF-2 are characterized by distinct cytokine profile.

(A-B) DSS colitis was induced in WT mice harboring SPF-1, cDysM or cSPF-2 communities. Colon samples were collected at day 7 of DSS and divided into two parts as proximal (PC) and distal (DC) colon. Cytokines (A) and chemokines (B) were measured using Multiplex/LEGENDplex kit from colonic tissue homogenates. Data represent n=7-17 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a nonparametric Kruskal-Wallis test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Levels of the pro-inflammatory cytokines IL-6 and IL-17A were significantly higher in the distal colon of both cDysM and cSPF-2 mice compared to SPF-1 mice (Figure 2.9A). Compared to SPF-1 and cDysM, colitis induced in cSPF-2 mice was distinctively characterized by higher levels of IFN-γ, IL-22 and TNF-α as well as lower levels of IL-18 mainly in the distal colon (Figure 2.9A). No changes were observed in IL-2, IL-4, IL-5, IL-10, IL-13 between the three microbiota communities (data not shown). In line with our previous observations [20], higher levels of the chemokine CCL5 were detected in the proximal colon of cDysM mice compared to SPF-1 and cSPF-2 mice (Figure 2.9B). In contrast, several other chemokines including LIX and KC, which recruit and activate neutrophils, along with MIP-1a and MIP-1b were significantly increased during colitis induced by cSPF-2 (Figure 2.9B).

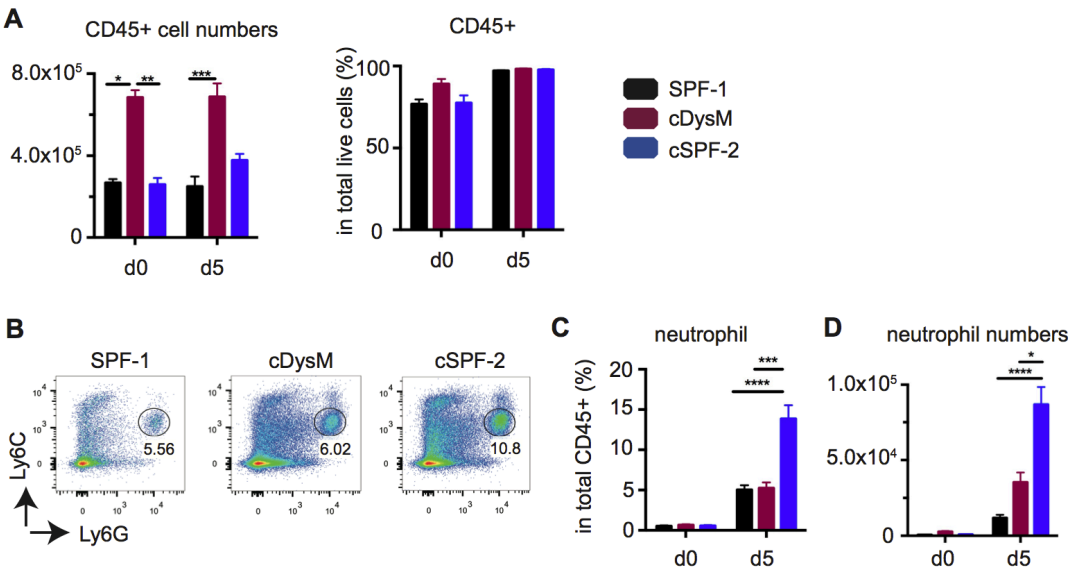


Figure 2.10 Increased neutrophil infiltratioin in SPF-2 mice during DSS colitis.

(A-D) Colonic lamina propria leukocytes (cLPL) were isolated from WT mice harboring SPF-1, cDysM or cSPF-2 microbiota during steady state (d0) and on d5 after DSS induction and analyzed by FACS. Total number of CD45+ cells in cLPL (A). Analysis of neutrophil infiltration upon DSS induction. Representative FACS plots showing frequencies of neutrophils (B). Frequencies (C) and total numbers (D) of neutrophils at d0 and d5 DSS. Data represent n=6-14 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a nonparametric Kruskal-Wallis test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

In parallel, we analyzed lamina propria leukocytes (LPLs) from colonic tissue by flow cytometry to identify whether distinct immune cell subsets are associated with disease induced by SPF-1, cDysM and cSPF-2 communities. Indeed, 2-fold increased numbers of CD45+ cells were observed in cDysM WT mice compared to SPF-1 and cSPF-2 WT mice both before and 5 days after induction of DSS colitis (Figure 2.10A). In line with the enhanced levels of neutrophil-attracting chemokines, colitis in cSPF-2 mice was associated with a specific increase in the relative abundance and total number of neutrophils (Figure 2.10B, 2.10C and 2.10D).

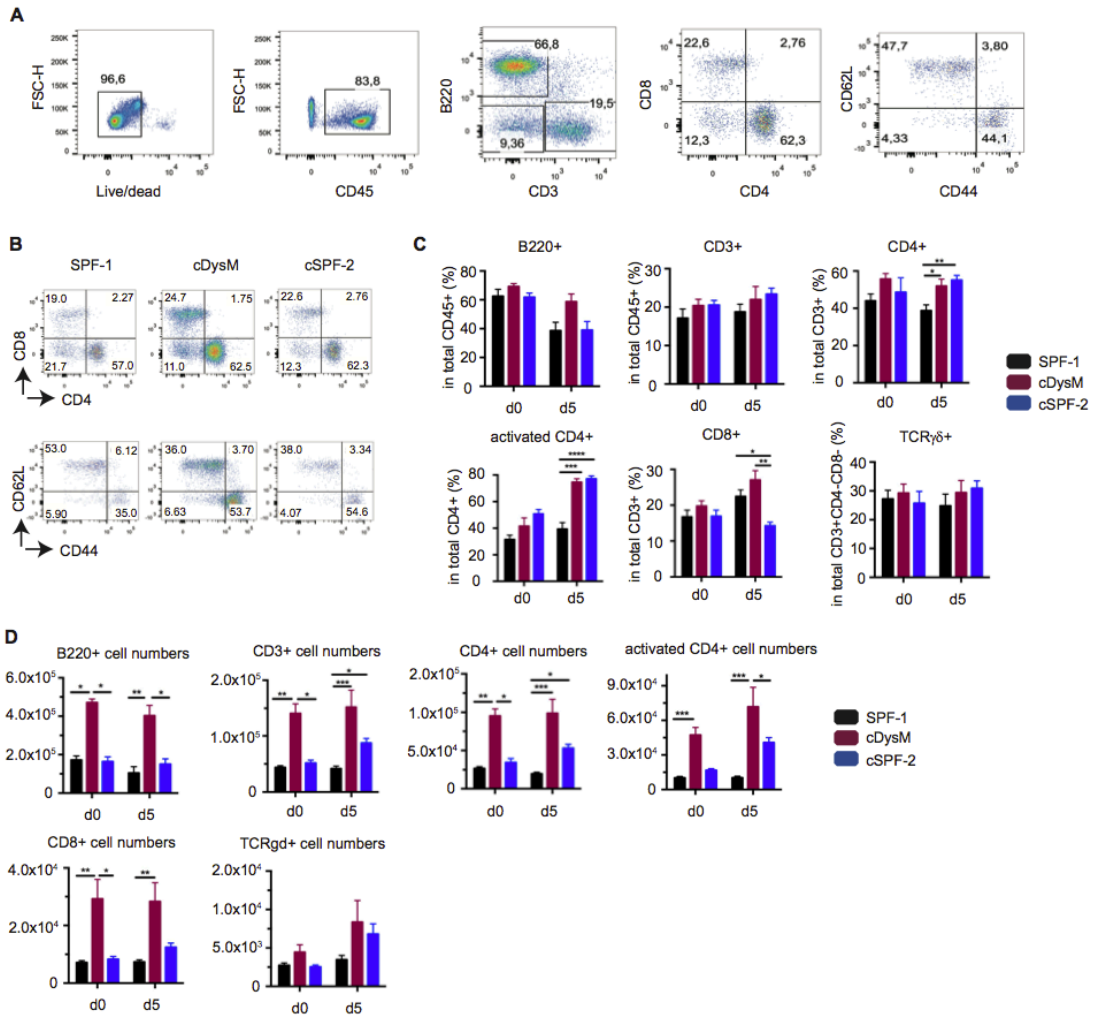


Figure 2.11 Colitis driven by DysM is characterized by distinct infiltration of adaptive immune cells.

(A-D) Colonic lamina propria leukocytes (cLPL) were isolated from WT mice harboring SPF-1, cDysM or cSPF-2 communities during steady state (d0) and d5 of DSS colitis and analyzed by FACS. Gating strategy of FACS data to analyze different adaptive immune cells (A). Representative FACS plots showing CD4 and CD8 frequencies gated on CD3+ cells and frequencies of naïve and activated CD4+ T cells during steady state (B). Frequencies of different immune cells at d0 and d5 of DSS (C). Total numbers of indicated immune cell subsets at d0 and d5 of DSS (D). Data represent n=5-17 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a nonparametric Kruskal-Wallis test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Despite similar frequencies of immune cell subsets of the adaptive immune system (Figure 2.11A, 2.11B and 2.11C), significant increases in the numbers of B220+ B cells and CD3+ T cells were observed before and after induction of DSS colitis in cDysM mice (Figure 2.11D). Increases in the numbers of CD4+ and CD8+ T cells but not γδ T cells contributed to this difference (Figure 2.11D). During, but not before DSS colitis, a higher frequency of CD4+ T cells in the colon of cDysM and cSPF-2 mice displayed an activated phenotype (Figure 2.11C). Notably, absolute numbers of activated CD4+ T cells were only increased in cDysM mice, both before and after induction of DSS colitis (Figure 2.11D). These analyses show that two colitogenic communities trigger distinct inflammatory immune pathways, i.e. enhanced neutrophil recruitment and pathogenic adaptive immune cell responses during DSS colitis.

2.4.5 αβ T cells trigger DysM but not SPF-2 mediated colitis development

In order to investigate which type of pathogenic adaptive immune responses contribute to disease exacerbation after colonization with the DysM community, we decided to compare severity of DSS colitis in WT as well as B or T cell deficient mice in SPF-1 and cDysM conditions. To assure comparable microbiota composition in WT and gene-deficient mice at baseline, all gene-deficient mouse lines were initially rederived into SPF-1 conditions using embryo transfer. Indeed, analysis of β diversity in the fecal microbiota demonstrated that SPF-1 communities in WT and gene-deficient mice were indistinguishable (Galvez E et al., manuscript submitted). To then generate experimental cohorts of WT and gene-deficient mice, the DysM microbiota was transferred into SPF-1 recipients using FT or cohousing and composition of the fecal microbiota was recorded before induction of disease. To investigate an involvement of T and B cells, we studied SPF-1 and cDysM *Tcrbd*$^{-/-}$ and *muMT*$^{-/-}$ mice, respectively.

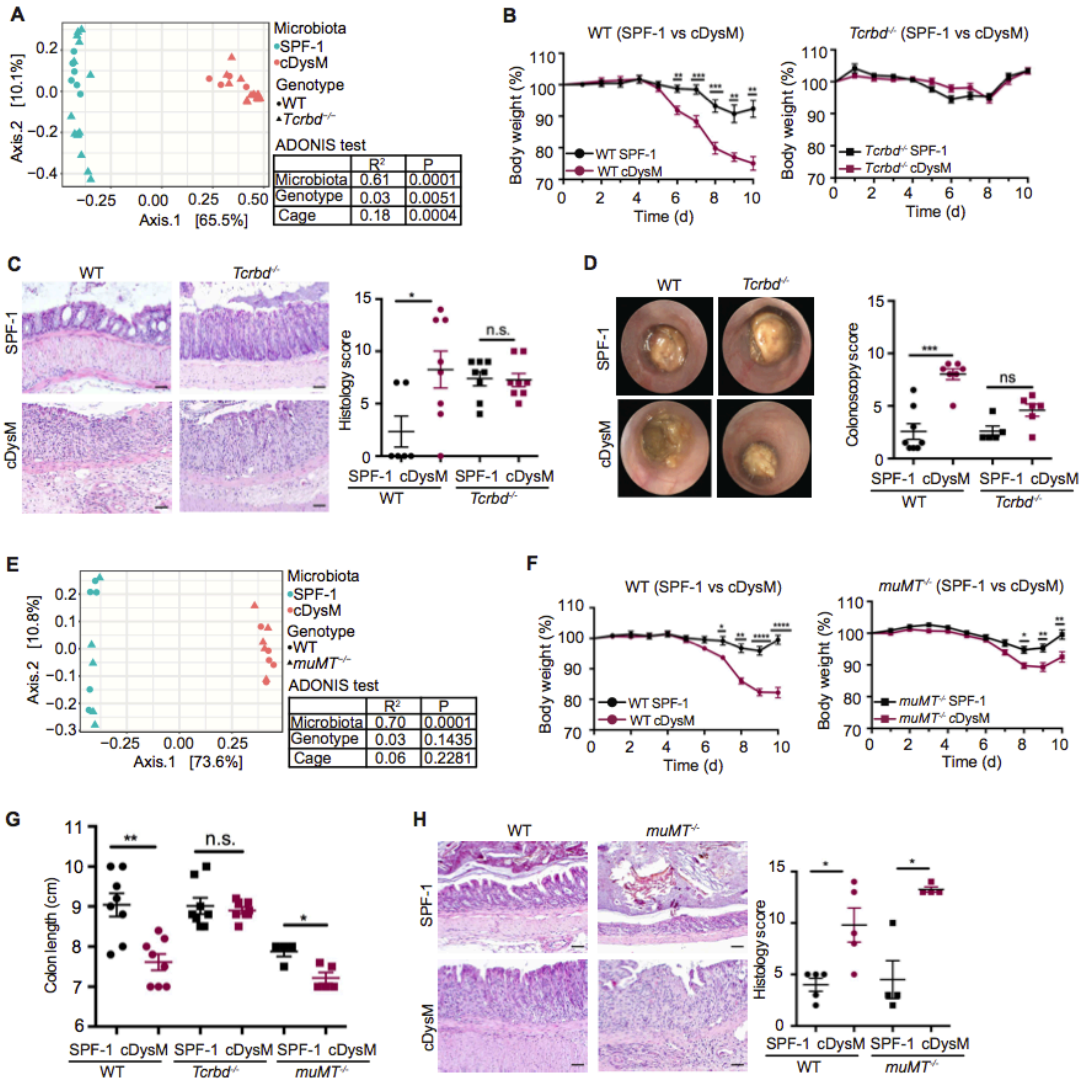
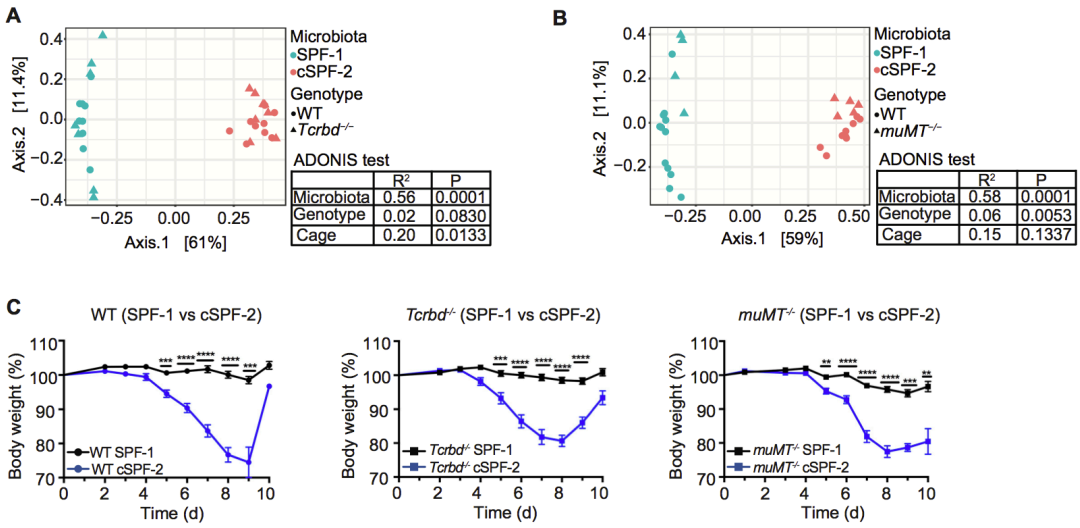


Figure 2.12 T cells are required for DysM mediated colitis.

(A-H) SPF-1 WT, *Tcrbd*$^{-/-}$ and *muMT*$^{-/-}$ mice were cohoused with DysM donor mice. Fecal microbiota composition was analyzed by 16S rRNA sequencing before induction of DSS colitis. Analysis of β-diversity (PCoA) of SPF-1 and cDysM WT and *Tcrbd*$^{-/-}$ mice (A) or *muMT*$^{-/-}$ mice (E) is shown along with multivariate analysis of variance (ADONIS test) of variables 'microbiota composition', 'genotype' and 'cage'. After DSS colitis induction body weight loss was measured in WT and *Tcrbd*$^{-/-}$ mice (B) or *muMT*$^{-/-}$ mice (F). Representative pictures and colitis severity score by colonoscopy performed on day 6 after colitis induction in SPF-1 and cDysM WT and *Tcrbd*$^{-/-}$ mice (D). 5 days after colitis induction colon shortening was measured (G) and histological analysis of distal colon was performed. Representative pictures of H&E-stained colon sections and scores in WT and *Tcrbd*$^{-/-}$ mice (C) or *muMT*$^{-/-}$ mice (H). Bar represents approx. 50µm. Data represent n=5-26 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Comparison of microbiota composition and multi-variate analysis before start of DSS colitis revealed that mice clustered according to SPF-1 and cDysM communities with genotype contributing only little (i.e. 3 %) to differences in microbiome composition (Figure 2.12A and 2.12E). Despite similar transfer of DysM into WT and *Tcrbd*$^{-/-}$ mice, strikingly, no differences in severity of DSS colitis was observed between SPF-1 and cDysM *Tcrbd*$^{-/-}$ mice as indicated by similar weight loss, unlike in WT mice that showed microbiota-modulated disease severity (Figure 2.12B). An involvement of T cells in transferring exacerbated disease severity was further corroborated by analyzing intestinal inflammation using histology (Figure 2.12C) and endoscopy (Figure 2.12D) as well as quantifying colon shortening (Figure 2.12G) of WT and *Tcrbd*$^{-/-}$ mice. In contrast to WT mice, deficiency in T cells resulted in no detectable differences in these parameters between SPF-1 and cDysM *Tcrbd*$^{-/-}$ mice. Transfer of the DysM community in SPF-1 *muMT*$^{-/-}$ mice resulted in an exacerbation of DSS colitis severity as indicated by significantly enhanced weight loss (Figure 2.12F), colon shortening (Figure 2.12G) and heightened intestinal inflammation (Figure 2.12H) compared to SPF-1 *muMT*$^{-/-}$ mice suggesting a limited involvement of B cells in colitis exacerbation.



ADONIS test	R^2	P
Microbiota	0.56	0.0001
Genotype	0.02	0.0830
Cage	0.20	0.0133

ADONIS test	R^2	P
Microbiota	0.58	0.0001
Genotype	0.06	0.0053
Cage	0.15	0.1337

Figure 2.13 T or B cells are not required for SPF-2 mediated colitis.

(A-C) SPF-1 WT, *Tcrbd*$^{-/-}$ and *muMT*$^{-/-}$ were cohoused with SPF-2 donor mice. Fecal microbiota composition was analyzed by 16S rRNA sequencing before induction of DSS colitis. Analysis of β-diversity (PCoA) of SPF-1 and cSPF-2 WT and *Tcrbd*$^{-/-}$ mice (A) or *muMT*$^{-/-}$ mice (B) is shown along with multivariate analysis of variance (ADONIS test) of variables 'microbiota composition', 'genotype' and 'cage'.
(C) DSS colitis was induced in SPF-1 and cSPF-2 WT, *Tcrbd*$^{-/-}$ and *muMT*$^{-/-}$ mice. Body weight was monitored over 10 days after DSS induction. Data represent n=5-15 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

To investigate, whether T cells are also required for disease exacerbation by the colitogenic SPF-2 microbiota, we introduced the SPF-2 community into SPF-1 WT, *Tcrbd*$^{-/-}$ and *muMT*$^{-/-}$ mice. After confirming that the fecal microbiota of mice clustered according to their microbial communities and not by genotype (Figure 2.13A, 2.13B), we induced DSS colitis. As expected from the results with Rag2-deficient mice, deficiency in B or T cells alone did not affect the transfer of heightened disease severity by cSPF-2 (Figure 2.13C).

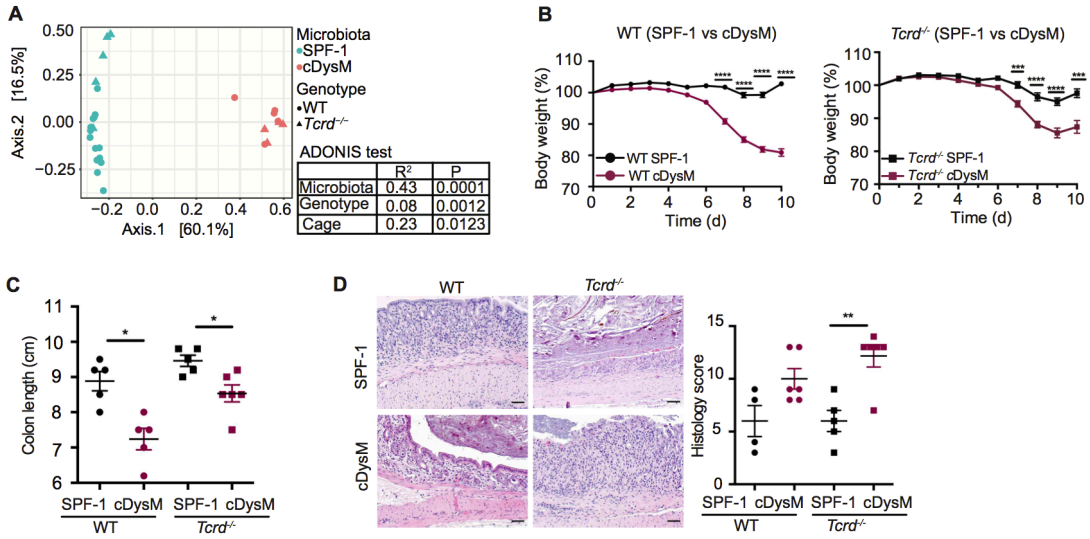


Figure 2.14 αβ T cells are required for DysM-mediated colitis.

(A-D) SPF-1 WT and *Tcrd*$^{-/-}$ mice were cohoused with DysM donor mice. Analysis of b-diversity (PCoA) of fecal microbiota from SPF-1 and cDysM WT and *Tcrd*$^{-/-}$ mice (A) is shown along with multivariate analysis of variance (ADONIS test) of variables 'microbiota composition', 'genotype' and 'cage'. After DSS induction body weight was measured daily (B). 5 days after colitis induction colon lengths of WT and *Tcrd*$^{-/-}$ mice were measured (I) and histology analysis of distal colon was performed. Representative pictures of H&E-stained colon sections and histology scores (D). Bar represents approx. 50µm. Data represent n=4-19 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

γδ T cells have been implicated in colonic tissue repair [40], hence, we next characterized DSS colitis severity in SPF-1 and cDysM *Tcrd*$^{-/-}$ mice. Notably, characterization of fecal microbiota demonstrated that mice clustered according to SPF-1 and cDysM microbiota (Figure 2.14A). Similar to what we observed in WT mice, transfer of DysM microbiota induced in *Tcrd*$^{-/-}$ mice enhanced weight loss (Figure 2.14B), colon shortening (Figure 2.14C) and heightened intestinal inflammation (Figure 2.14D). From these results we concluded that T cells are essential for DysM but not SPF-2 induced exacerbation of disease. Specifically, our data suggests that modulation of αβ T cells by members of the DysM community is important. Finally, we exclude a major contribution of B cells to DysM-mediated colitis.

2.4.6 Pathogenic CD4+ T cells are crucial to induce DysM-mediated colitis

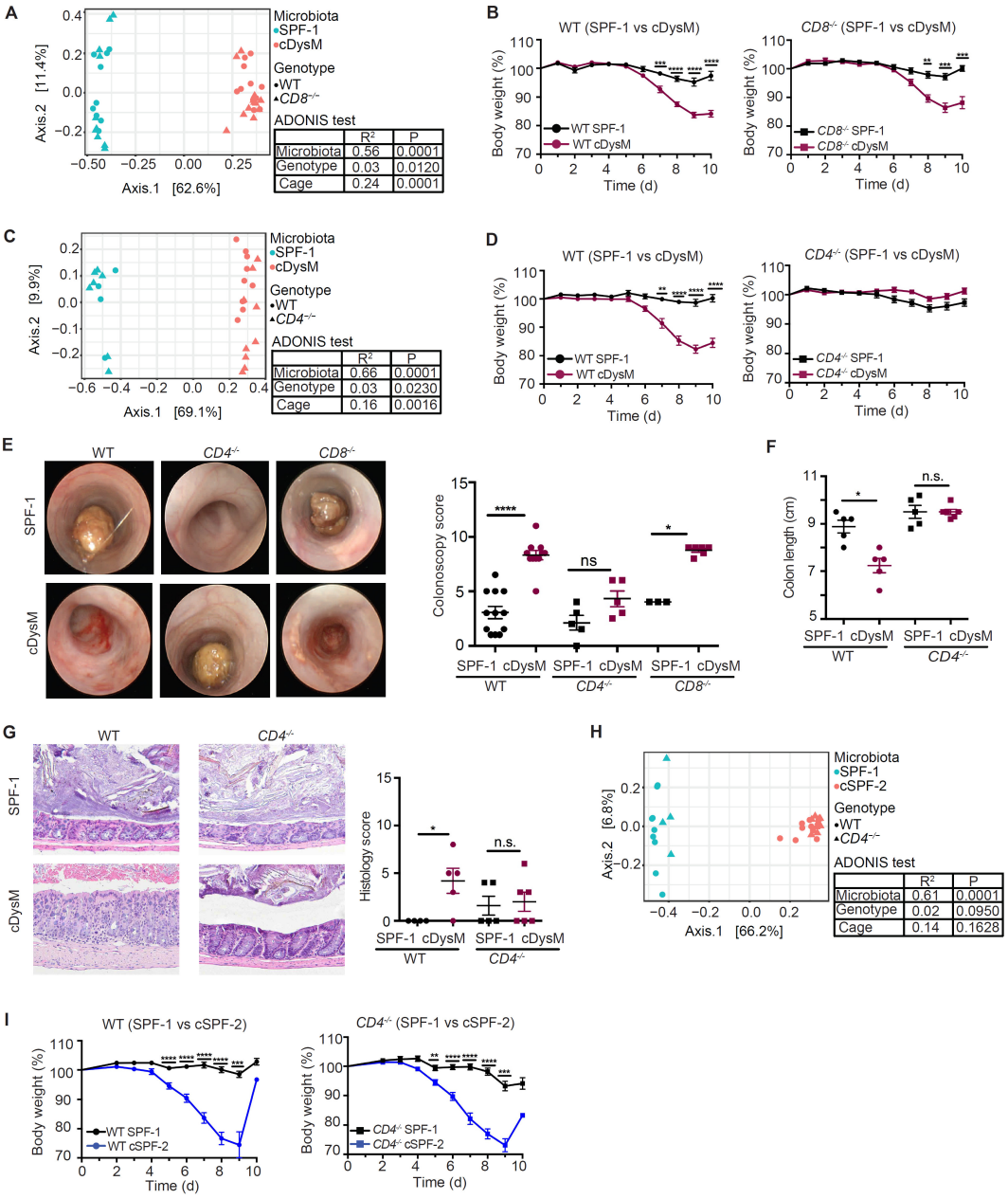


Figure 2.15 CD4+ T cells are crucial to develop DysM, but not SPF-2 mediated colitis.

(A-G) SPF-1 WT, *CD8*$^{-/-}$ and *CD4*$^{-/-}$ mice were cohoused with DysM donor mice and 16S rRNA sequencing of fecal microbiota was performed before induction of DSS colitis.

CD4+ but also CD8+ T cells contribute to different aspects of intestinal homeostasis and inflammation [5]. To specifically probe an involvement of these T cell subsets, we decided to compare DSS colitis severity in SPF-1 and cDysM *CD4*$^{-/-}$ and *CD8*$^{-/-}$ mice. Fecal microbiota of mice clustered again according to SPF-1 and cDysM but not to genotype (Figure 2.15A and 2.15C). After DSS induction, *CD8*$^{-/-}$ but not *CD4*$^{-/-}$ showed enhanced weight loss and colitis severity after DysM transfer comparable to WT mice (Figure 2.15B, 2.15D and 2.15E). Furthermore, analysis of intestinal inflammation using histology (Figure 2.15G) and quantification of colon shortening (Figure 2.15F) in SPF-1 and cDysM WT and *CD4*$^{-/-}$ mice corroborated that CD4+ but not CD8+ T cells are required for DysM-induced exacerbation of disease. To evaluate whether CD4+ T cells contribute to enhanced colitis severity by the colitogenic SPF-2 microbiota, we introduced the SPF-2 community into SPF-1 WT and *CD4*$^{-/-}$ mice. Analysis of fecal microbiota of mice confirmed clustering according to microbiota and not by genotype (Figure 2.15H). Deficiency in CD4+ T cells did not affect the transfer of heightened disease severity (Figure 2.15I), further supporting that SPF-2 drives colitis severity irrespective of T cells.

Figure 2.15 (cont.) Analysis of β-diversity (PCoA) of SPF-1 and cDysM WT and *CD8*$^{-/-}$ mice (A) or *CD4*$^{-/-}$ mice (C) is shown along with multivariate analysis of variance (ADONIS test) of variables 'microbiota composition', 'genotype' and 'cage'. After DSS colitis induction body weight loss was measured in WT and *CD8*$^{-/-}$ mice (B) or *CD4*$^{-/-}$ mice (d). Representative pictures and colitis severity score by colonoscopy performed on day 6 after colitis induction (E). 5 days after colitis induction colon shortening was measured (F) and histological analysis of distal colon was performed in WT and *CD4*$^{-/-}$ mice. Representative pictures of H&E-stained colon sections and histology scores (G). Bar represents approx. 50µm.
SPF-1 WT and CD4-/- mice were cohoused with SPF-2 donor mice and 16S rRNA sequencing of fecal microbiota was performed before induction of DSS colitis. Analysis of β-diversity (PCoA) is shown (H). Body weight was monitored (I). Data represent n=5-20 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

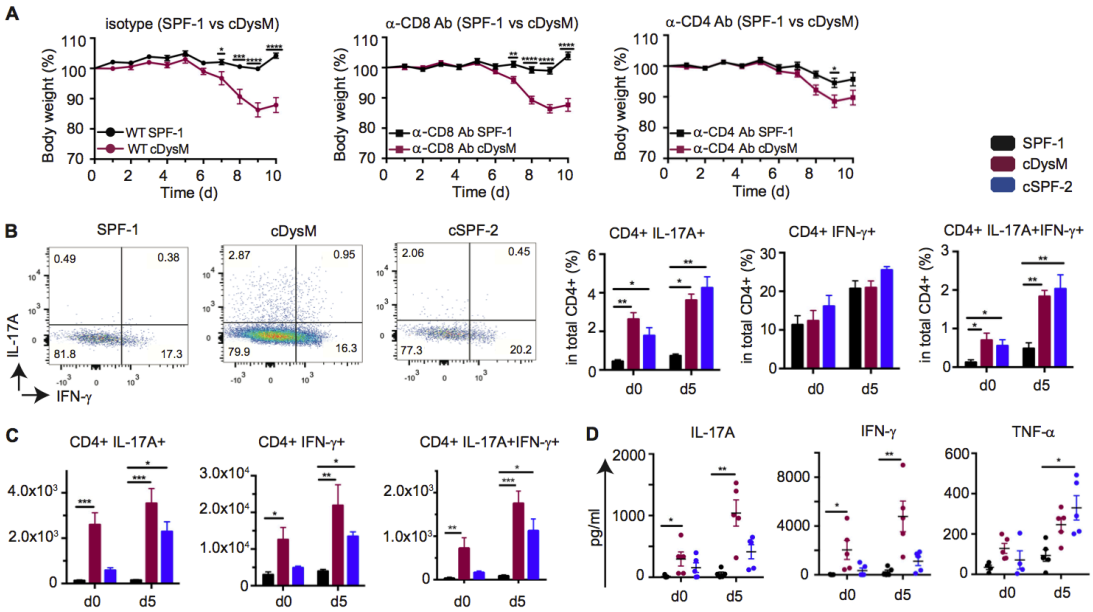


Figure 2.16 Distinct pathogenic CD4+ T cells are crucial for DysM to enhance colitis severity.

SPF-1 and cDysM WT mice were injected with isotype control, anti-CD8 or anti- CD4 antibodies at d-1, d3 and d7 of DSS colitis and. body weight was compared (A). Representative pictures, frequencies (B) and total numbers (C) of CD4+ T cells producing IFN-γ and/or IL-17A from isolated cLPL from IL-17A^{GFP} IFN-γ^{Katushka} FoxP3^{RFP} triple reporter mice with different microbiota.

(D) cLPL were isolated from SPF-1, cDysM and cSPF-2 mice during steady state and on d5 after DSS colitis induction and restimulated with α-CD3/CD28 for 3 days. Cytokine levels were measured from supernatant. Data represent n=5-20 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test (A) and nonparametric Kruskal-Wallis test (B-D) *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

To investigate whether pathogenic CD4+ T cells are required during DysM-enhanced DSS colitis, we treated SPF-1 and cDysM WT mice during DSS colitis with isotype control antibody or depleting antibodies against CD4 or CD8, respectively. Depletion of CD4 expressing cells but not CD8 expressing cells resulted in a failure of the DysM community to exacerbate DSS colitis (Figure 2.16A), highlighting that CD4+ T cells are required during the development of DysM-enhanced colitis. Consequently, we extended our immunophenotyping and analyzed the production of proinflammatory cytokines in CD4+ T cells before and during DSS colitis. We initially focused on IFN-γ and IL-17 and hence, isolated cLPL from SPF-1, cSPF-2 and cDysM IL-17A^{GFP} IFN-γ^{Katushka} FoxP3^{RFP} triple reporter mice allowing the in situ monitoring of cytokine production [41]. Transfer of the DysM but not SPF-2 community resulted in enhanced numbers of IL-17A and IFN-γ single and IL-17A/IFN-γ double cytokine-producing CD4+ T cells already before induction of DSS colitis (Figure 2.16B and 2.16C). After induction of DSS colitis, enhanced numbers of cytokine producing CD4+ T cells were observed in mice with both colitogenic communities (Figure 2.16B and 2.16C). In addition to monitoring cytokine production in situ, we isolated cLPL from SPF-1, cSPF-2 and cDysM mice before and after induction of DSS colitis and stimulated them with αCD3 and αCD28 to quantify cytokine production from T cells. Strikingly, T cells from cDysM mice produced larger amounts of IL-17A and IFN-γ than T cells from SPF-1 and cSPF-2 mice (Figure 2.16D) both during steady state and colitis. Notably, TNF-α production after restimulation of T cells was highest during colitis in mice colonized with SPF-2 (Figure 2.16D).

To further investigate the ability of the DysM to drive T cell-mediated intestinal inflammation, we transferred CD45RB(high)Foxp3-CD4+ T cells from IL-17A^{GFP}IFN-γ^{Katushka} FoxP3^{RFP} triple reporter mice into SPF-1, cSPF-2 and cDysM *Rag2*$^{-/-}$ mice.

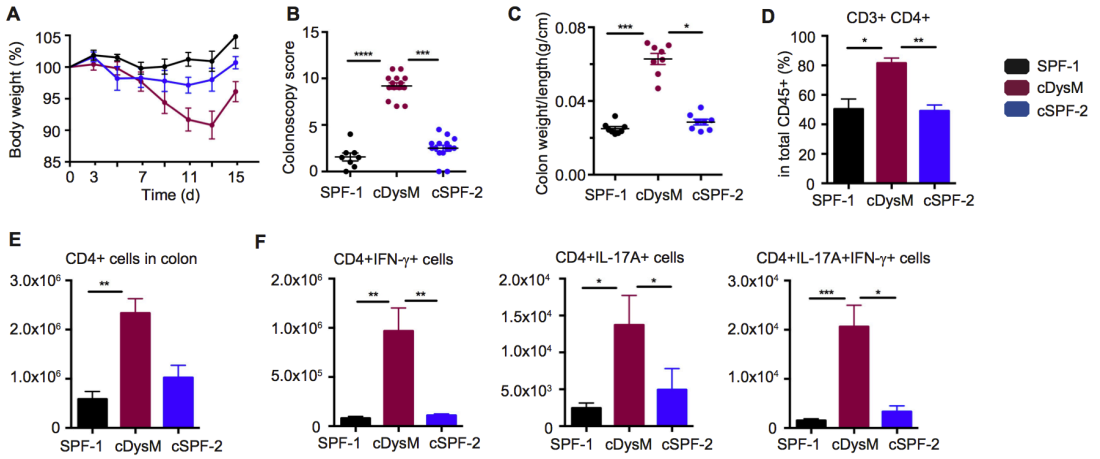


Figure 2.17 DysM, but not SPF-2 can enhance T-cell driven colitis severity.

(A-F) T cell transfer colitis was induced by injecting CD4+Foxp3-CD45RB(high) T cells into SPF-1, cDysM or cSPF-2 *Rag2*$^{-/-}$ recipients. Body weight was measured after T cell transfer (A). Colonoscopy severity score at d14 post transfer (B). On d16 post injection mice were sacrificed. Colon weight/length ratio (C) was measured. Frequencies (D) and total numbers (E) of CD4+ cells in cLPL were monitored by FACS. Infiltration of different cytokine producing cell population in colon is displayed (F). Data represent n=7-16 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a nonparametric Kruskal-Wallis test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Already after two weeks, when mice differed only mildly in their weight loss (Figure 2.17A), we observed already significantly higher intestinal inflammation as quantified by colonoscopy in cDysM compared to SPF-1 and cSPF-2 recipients (Figure 2.17B). Accordingly, cDysM mice displayed an enhanced colon weight to length ratio and cellular infiltration (Figure 2.17C 2.17D and 2.17E). Specifically, IFN-γ+ CD4+ T cells numbers were significantly increased (Figure 2.17F). While numbers of IL-17A+ and double cytokine producing T cells were also significantly enhanced in cDysM colonized mice, their total numbers were much lower than those of IFN-γ+ CD4+ T cells (Figure 2.17F). Taken together, this demonstrates that the DysM induces pathogenic CD4+ T cells producing high levels of proinflammatory cytokines. Moreover, these microbiota-induced cells are essential to drive disease in two distinct colitis models.

2.4.7 Recognition of antigens from dominant microbial members by CD4+ T cells drives DSS colitis severity in DysM mice

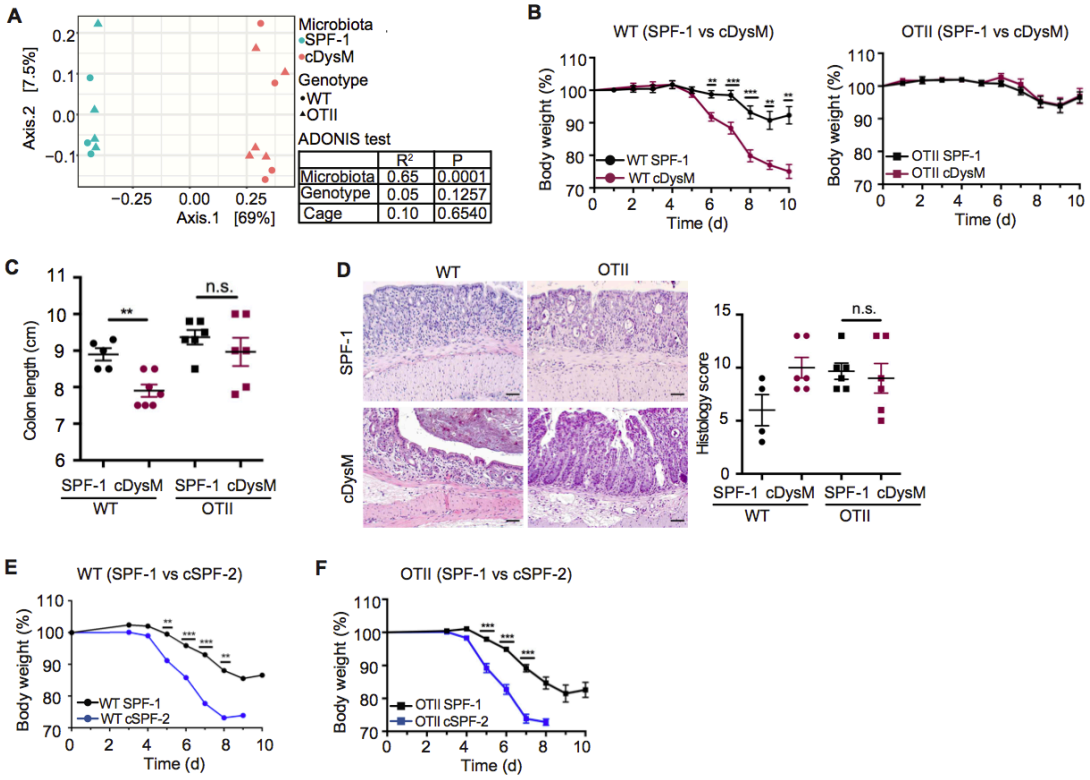


Figure 2.18 Antigen recognition by CD4+ T cells is indispensible for DysM, but not SPF-2 driven colitis.

(A-B) SPF-1 WT and OTII transgenic mice were cohoused with DysM donor mice and DSS colitis was induced. Analysis of β-diversity (PCoA) of fecal microbiota is shown (A). Body weight (B) and intestinal inflammation by measuring colon length (C) and histology (D) on d5 of DSS colitis were monitored. Representative pictures of H&E-stained colon sections, bar represents approx. 50µm. (E-F) DSS colitis was induced in SPF-1 and cSPF-2 WT and OTII transgenic mice and body weight was monitored for 10 days. Data represent n=5-20 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Intestinal CD4+ T cells recognize diet-derived and microbial antigens and this process is important in intestinal homeostasis and inflammation. To investigate whether recognition of microbial antigens by CD4+ T cells is required for exacerbation of colitis in DysM mice, DSS colitis was induced in OT-II transgenic mice colonized with the SPF-1, cSPF-2 or cDysM communities (Figure 2.18A). Strikingly, cDysM OT-II mice did not display

exacerbation of DSS colitis severity as indicated by the lack of DysM transfer induced changes in body weight loss, intestinal inflammation and colon shortening (Figure 2.18B, 2.18C and 2.18D). In contrast, cSPF-2 OT-II mice were characterized by similar weight loss compared to cSPF-2 WT mice (Figure 2.18E and 2.18F). This shows that antigen-specificity of CD4+ T cells is required for modulation of disease-severity by the DysM but not SPF-2 communities.

While both DysM and SPF-2 communities trigger severe colitis in the host the mechanisms of pathogenesis are completely opposing. Consequently, we wanted to understand whether triggering of innate or adaptive immunity by the SPF-2 or DysM communities dominates over each other when cotransferring them into SPF-1 recipients. Analysis of microbiota composition in recipient mice after cohousing of SPF-1 recipient as well as SPF-2 and DysM donor mice showed that the resulting community largely resembled the cDysM community (Figure 2.19A). Accordingly, host gene expression signatures in cDysM+SPF-2 mice were similar to the ones observed in cDysM mice including upregulation of genes associated with T cell, B cell, cytokine and chemokine signaling as well as upregulation of *Cd4* (Figure 2.19B, 2.19C, 2.19D, and 2.19E). Moreover, mice with cDysM+SPF-2 displayed high weight loss, intestinal inflammation, colon shortening and mortality compared to SPF-1 mice (Figure 2.19F, 2.19G and 2.19H). Strikingly, the cDysM+SPF-2 community failed to induce severe colitis in *CD4*$^{-/-}$ mice (Figure 2.19I, data not shown). These results demonstrate that the DysM community and its pathogenesis mechanism, i.e. the induction of pathogenic antigen-specific CD4+ T cell responses, dominates over SPF-2 induced changes during colitis induction.

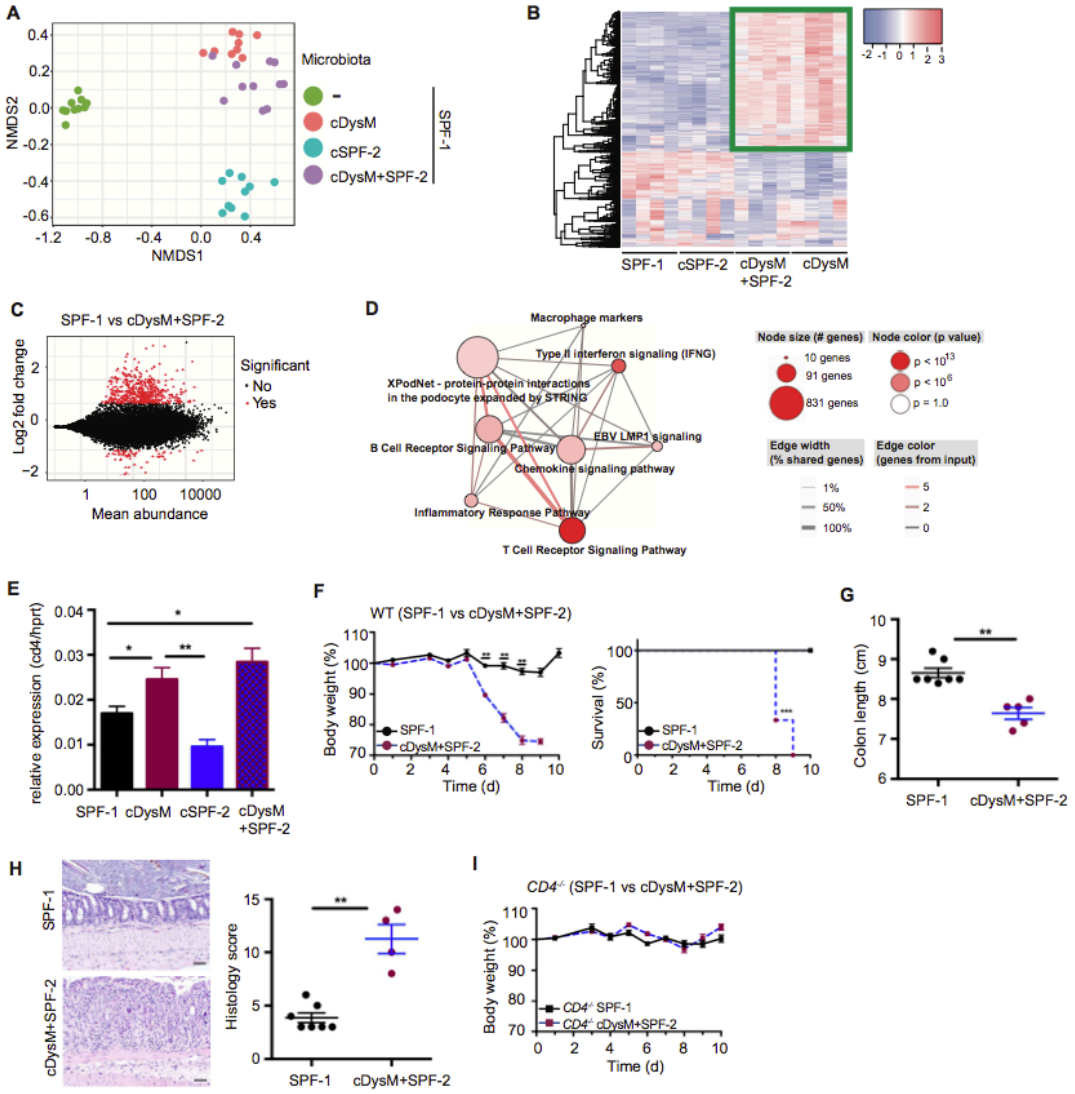


Figure 2.19 CD4+ T cells drives DSS colitis severity in DysM mice by recognizing antigens from dominant microbial members.

(A-I) SPF-1 WT mice were cohoused with DysM, SPF-2 or both DysM and SPF-2 donor mice, respectively. β-diversity analysis (PCoA) of fecal microbiota (A). RNAseq analysis was done from total colonic tissue. Heatmap shows quantification of RNA reads (B) and DEseq analysis to identify significant up/down-regulation (fold change >2) of genes in SPF-1 and cDysM+SPF-2 conditions (C). Pathway analysis based on GO terms of genes significantly upregulated (2-fold) in cDysM+SPF-2 compared to SPF-1 mice (D). Relative *Cd4* expression in colonic tissue (E). After induction of DSS colitis in WT mice body weight loss and survival was monitored (F) and intestinal inflammation was measured by colon shortening (G) and histological analysis (H). Representative pictures of H&E-stained colon sections. Bar represents approx. 50µm. SPF-1 *CD4*$^{-/-}$ mice were cohoused with DysM and SPF-2 donor mice and DSS colitis was induced (I). Data represent n=5-12 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent an unpaired Student's t test. *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

2.5 Discussion

The incidence of IBD, a pathological immune response against microbial and environmental antigens, is gradually rising in developed countries, but no specific curative therapies exist. While several new therapies have been introduced in the past years, it remains to be investigated, which subgroups of IBD patients will benefit from these novel drugs [42]. Alterations in the microbiome have been hypothesized to contribute to the development of IBD and patient data suggests the existence of microbial signatures associated with specific disease entities such as CD [6,37]. However, it remains in question whether those changes are causal and can be potentially used to improve selection of IBD therapy or rather are the result of ongoing inflammation that alters the intestinal microenvironment [43]. Here we identified and then characterized distinct types of microbial communities that directly affect the severity of intestinal inflammation in an immunocompetent host. We showed that these communities alter disease susceptibility via opposing mechanisms, one requiring antigen-specific CD4+ T cell responses and the other being mediated by innate immune cells (Figure 2.20). Our results provide novel insight into host-microbiota interactions directly responsible for altering susceptibility to IBD and suggest that microbiome signatures could be employed to predict disease pathogenesis.

Healthy human individuals can differ greatly in the composition of their intestinal microbiota, but despite this variability alterations in the microbiota of patients have been associated with different types of human diseases [8,9]. Germfree mice have been fundamental to address the causal role of alterations of the microbiome in mouse models of human disease. Likewise, conventionally housed laboratory mice that feature tremendous differences in the microbiome represent a valuable resource to study the contribution of diverse microbial ecosystems to disease development [44]. But the observed variability within conventionally housed mouse lines requires sophisticated experimental planning to control and document this potentially cofounding factor in animal studies. In a first effort to reduce experimental variability, the concept of specific pathogen free housing conditions was introduced to

exclude unwanted influences imposed by the presence of potential pathogens such as *Helicobacter spp.* or mouse norovirus commonly present in wild and conventionally housed mice [44]. Yet, microbiota composition differs greatly between SPF mice from different commercial breeders and academic institutions [45] and those differences influence host responses, e.g. the presence of Th17 cells in SFB colonized mice [36] or the lowered susceptibility to malaria infection as a consequence of increased abundance of Lactobacillaceae and Bifidobacteria [46]. These observations also make genetically identical SPF mice a versatile experimental model to explore diverse microbial communities and to study host - microbiota interactions in health and disease.

A common feature in IBD, particularly in UC, is an impairment of the intestinal barrier resulting in enhanced exposure to luminal microbes. By employing a mouse model of damage to the intestinal barrier, DSS colitis, we demonstrate that isogenic SPF mice with differences in microbiome composition feature altered susceptibility to intestinal inflammation. These alterations in colitis susceptibility were reminiscent of the dominant effects exerted by the microbial communities observed in conventionally housed Nlrp6 inflammasome deficient mice (DysM)[20]. Specifically, we noted that transfer of colitogenic communities into mice relatively resistant to induction of DSS colitis is sufficient to alter disease susceptibility even in immunocompetent mice. Upon induction of disease, both the DysM community, as well as the SPF-2 community, induced severe colitis compared to the relative resistant SPF-1 community, yet, the mechanisms of pathogenesis differed strongly. While macroscopic characterization of inflammation did not reveal significant differences in mice with colitogenic communities, measurements of cytokine and chemokine signatures, as well as immunophenotyping, revealed large differences. Inflammation in SPF-2 mice was characterized by high levels of TNF-α and neutrophil-attracting chemokines coinciding with significant higher infiltration of neutrophils into the inflamed tissue. In line with previous findings, DysM mice featured higher levels of the chemokine CCL5, known to attract innate and adaptive immune cells carrying CCR1, CCR3, CCR4 and CCR5 [20]. Here, we identified high infiltration of activated CD4+ T cells in DysM mice

hinting towards a potential involvement of these cells in the intestinal pathogenesis. The hypothesis of adaptive immune cells being involved in DysM mice was further corroborated by the observation that extended colonization with the DysM, but not SPF-2 community, was required to transfer the disease susceptibility. Subsequently, we evaluated the effect of the transfer of the two colitogenic communities in mice lacking specific subsets of adaptive immune cells. For these comparisons we employed WT and gene-deficient mice that were embryo-transferred into our vivarium using SPF-1 foster mothers resulting in a standardized microbiota (Galvez E et al., manuscript submitted). Moreover, we included cohousing of WT and gene-deficient mice to further reduce microbiota variability within experiments and documented for all experiments microbiota composition using 16S rRNA sequencing. Using this carefully controlled approach, we observed significant increases in IL-17A and IFN-γ secretion by CD4+ T cells during DysM and SPF-2 driven colitis. Notably, this is in line with an association of CD4+ T cells and proinflammatory cytokines including IL-17, IFN-γ, and IL23 with human IBD [1]. Strikingly, our experiments demonstrated that CD4+ T cells are only essential to mediate the exacerbation of DSS colitis in DysM but not SPF-2 mice. In contrast, despite measurable CD4+ T cell activation during DSS colitis, SPF-2 modulated disease severity independent of adaptive immune cells. TCR mediated recognition of cognate antigens is required for proper T cell function and recognition of microbial antigens has been suggested to significantly contribute to development of colitis [47]. Using OTII transgenic mice we could show that DysM-driven but not SPF-2-driven colitis development strongly depended on the presence of antigen-specific CD4+ T cells. The presence of *in vivo* cytokine secreting CD4+ T cells already before induction of DSS colitis in DysM mice suggests that colonic CD4+ T cells recognize cognate microbial antigens already during this phase, similar to what has been observed for SFB-specific CD4+ T cells in the small intestine [48]. Importantly, antibody-mediated depletion of CD4+ T cells during colitis resulted in failure to transfer enhanced colitis susceptibility. This demonstrated that to enhance colitis modulation of the mucosal barrier by CD4+ T cells in the steady state was not sufficient and rather required the

presence and presumably the effector functions of CD4+ T cells during colitis. The distinct property of the DysM community to prime and activate pathogenic CD4+ T cell responses was further corroborated using a model for CD4+ T cell-mediated colitis. Specifically, transfer of CD4+ T cells in *Rag2*$^{-/-}$ mice harboring the DysM but not the SPF-2 microbiota enhanced intestinal inflammation and cytokine production by CD4+ T cells. Together this shows that colitogenic communities exert their pathogenic effects in the same disease model by opposing mechanisms (Figure 2.20).

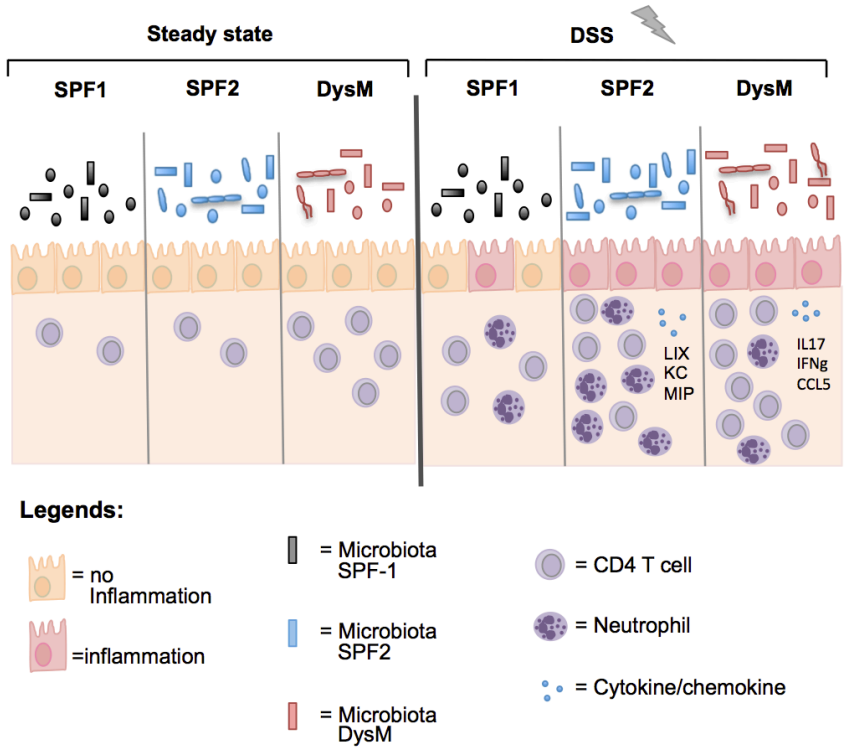


Figure 2.20 Distinct microbial communities drive colitis via opposing pathways.

Already at baseline, colonization with distinct microbial communities exerts different host immune responses in the intestinal mucosa (steady state). When both SPF-1 and SPF-2 community do not show any host responses, DysM colonization results in increased CD4+ T cell infiltration. Upon trigger with DSS, distinct microbial communities drive colitis via different pathways (DSS). SPF-1 community drives a mild colitis and both SPF-2 and DysM communities drive severe colitis. However, SPF-2 colitis enhancement is characterized by infiltration of neutrophils and associated chemokines, whereas, DysM mediated colitis requires antigen specific CD4+ T cells to enhance disease severity.

Different models have been proposed to explain the contribution of the microbiota to the development of IBD and other complex diseases. On one hand, specific members of the microbiota, so called pathobionts, have been proposed to play essential roles, i.e. their presence is sufficient to induce disease similar to the "one-microbe – one-disease" concept. Detailed characterization of the colitogenic communities using 16S rRNA sequencing revealed the varying presence of potential pathobionts such as SFB, *Prevotella spp*., *Helicobacter spp*., Enterobacteriaceae and Verrucomicrobiaceae in DysM and SPF-2 mice. SFB have been shown to modulate intestinal T cell immunity and systemic autoimmunity [36]. But, based on its presence in both SPF-2 and DysM mice, a role in driving the differential requirement for CD4+ T cells can be excluded. Similarly, members of the genus Prevotella, previously found to be enriched in the colitogenic microbiota of *Nlrp6*$^{-/-}$ mice [20], were present in both colitogenic communities indicating that they are not involved in regulating the different pathogenicity modes. Helicobacteriaceae have been demonstrated to induce the development of colitis in *Il10*$^{-/-}$ mice [13]. Notably, monocolonization of germfree *Il10*$^{-/-}$ mice with *H. hepaticus* failed to induce colitis, moreover, in some colonies of *Il10*$^{-/-}$ mice *H. hepaticus* colonization did not cause colitis, demonstrating that the ability of *H. hepaticus* and potentially other members of this family to induce colitis seems to depend very strongly on the composition of the microbiota. While Helicobacterceae were absent in SPF-2 mice, DysM mice harbored different members of this family including *H. typhlonius*, *H. rodentium* and *H. muridarum*, but did not harbor *H. hepaticus*. The role of these bacteria in modulating severity of DSS colitis and triggering of specific T cell responses in immunocompetent hosts is unknown. Finally, both Enterobacteriaceae and Verrucomicrobiaceae, specifically *Akkermansia muciphilia*, bloomed during the induction of DSS colitis in SPF-2 mice, yet it is being debated whether expansion during disease suggests a contribution to disease development or rather a consequence of the ability to utilize inflammation-induced metabolites. In contrast to the "one-microbe-one disease" model the concept of dysbiosis, an imbalance of the community, has been proposed for microbiome-mediated modulation of diseases [49]. One characteristic of

dysbiotic communities including those in IBD patients has been suggested to be an imbalance between Bacteroides, Firmicutes and Proteobacteria with an overexpansion of Bacteroides and Proteobacteria over Firmicutes [37]. Lowered Firmicutes / Bacteroides ratios were noted in all colitogenic communities including SPF-2 and DysM, whereas the ratios between Firmicutes and Proteobacteria (F/P) was not consistently different between susceptible and resistant groups. Notably, the F/P ratio was the lowest in DysM mice and according to our data this is associated with a distinct mode of pathogenicity. Whether in the cases of the SPF-2 and DysM community specific pathobionts or a general dysbiosis are responsible for driving distinct pathogenicity requires further investigation. Meanwhile these colitogenic communities provide an innovative approach to dissect the immune pathways involved in microbiota modulated colitis development and to test whether the efficacy of novel immunomodulators is influenced by the microbiome.

In summary, our data show how distinct microbial communities drive the development of intestinal inflammation in immunocompetent hosts by modulating opposing arms of the immune system. Our study suggests a concept that triggering of different immune pathways by microbial communities can alter disease susceptibility eventually resulting in similar host pathophysiology. This implies that a personalized immunomodulatory treatment according to distinct microbial signatures may be beneficial for IBD patients.

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CHAPTER 3

Cytokine knock-in reporter mice demonstrates SFB-dependent bystander effect on intestinal distinct CD4+ T cell subsets upon *Salmonella* infection

3.1 Summary

The mucosal immune system plays an essential role to protect the host against infections. Recent studies have established that in the gastrointestinal (GI) tract, the gut microbiota as a community contributes to antimicrobial immunity through modulation of innate and antigen-specific mucosal immune responses. However, which specific members of these diverse intestinal microbial communities are responsible for the immunomodulatory effects is less well understood. The best-studied bacterium, known as segmented filamentous bacteria (SFB), is a strong inducer of Th17 cells in the small intestine of mice during steady state. But, the diversity and functionality of SFB induced memory CD4+ T cells is still being debated. By employing novel gnotobiotic cytokine knock-in reporter mice (IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP}) we confirm that SFB colonization in the ileum is sufficient to induce the differentiation of Rorγt+ Th17 cells that subsequently seed the large and small intestine. Notably, only in the terminal ileum these cells produce IL-17A even in the steady state, likely as consequence of recognizing cognate SFB-derived antigens. Upon induction of intestinal inflammation by *Salmonella* Typhimurium infection, we observed IL-17A+IL-22+ and IL-17A-IL-22+ CD4+ T cells at early timepoints after infection only in the cecum of mice harboring SFB. Based on the enrichment of SFB-specific TCR Vβ chains in these "innate-like" CD4+ T cell subsets, we hypothesize that bystander activation induces cytokine production from microbiota-specific memory T cells rapidly after infection with enteropathogens. Supporting this conclusion, SFB monocolonization of germfree mice was sufficient to induce the appearance of similar subsets in steady state and after infection. Gene expression analysis

by RNAseq revealed distinct properties of SFB modulated T cell subsets in the steady and after infection including the differential expression of IL-17F and IFN-γ. Finally, SFB colonized mice displayed reduced pathogen invasion in the cecum suggesting that modulation of CD4 T cells by SFB is associated with improved immune defense.

In summary, we demonstrate that SFB induces Th17 and other CD4+ T cell subsets in the steady state, which serve as “innate-like” source of numerous cytokines rapidly after infection. These findings provide new insight into the previously known role of SFB to shape CD4+ T cells and may have implications for patients suffering from GI diseases, which are characterized by diverse subsets of cytokine-producing CD4+ T cells in the intestine.

3.2 Introduction

Antigen-specific CD4+ T cells, which are part of the adaptive immune system, play pivotal roles during immune responses through their ability to differentiate into diverse subsets of T helper (Th) cells and to subsequently secrete potent immunoregulatory cytokines as well as to form long-lasting memory. Th17 and Th22 are two subsets of CD4+ T cells and involved in maintaining the balance between homeostasis and inflammation at mucosal sites. Th17 cells are characterized by their production of effector cytokines interleukin (IL)-17A, IL-17F, IL-22 and function as important activators of innate immune effectors, thereby contributing to the mucosal defense against bacteria and fungi [1]. Pathogenic roles of Th17 cells have also been described in different animal models of autoimmune disease including rheumatoid arthritis, psoriasis, multiple sclerosis and inflammatory bowel disease [2–5]. In humans, Th22 cells, a newly classified CD4+ T cell subset distinct from Th17 cells due to their complete lack of IL-17A expression, are considered the major source of adaptive immune cell-derived IL-22 [6]. IL-22 confers both proinflammatory and/or tissue protective functions based on type of inflammatory response, cellular source and surrounding cytokine milieu [7,8]. Studies performed in different *in vivo* and *in vitro* models have shown a high heterogeneity of both Th17 and Th22 cell subsets characterized by the variable production of

additional immunomodulatory cytokines such as IFN-γ and IL-10. The variable impact of these cell subsets on the outcome of different disease models is likely related to their heterogeneity. Therefore, detailed studies are needed to identify the instructive networks as well as both molecular and functional properties of potentially heterogenous Th17 and Th22 cell subsets.

The making of lineage-decisions during T cell differentiation is heavily influenced by environmental stimuli. In the gastrointestinal (GI) tract hundreds of distinct species of microorganisms constantly convey signals to the host mucosal immune system. However, it is evident now that only specific members of the intestinal microbiota are able to induce antigen-specific helper CD4+ T cells in absence of a classical infection. The best-known example is a bacterium known as segmented filamentous bacteria (SFB), which is closely related to family Clostridiaceae and induces the maturation of the mucosal immune system in the small intestine, e.g. it drives the differentiation of Th17 cells [9]. A further study demonstrated that SFB-triggered cascade, in which innate lymphoid cell (ILC) -3 secretion of IL-22 is crucial for production of serum amyloid A (SAA) proteins 1 and 2 from intestinal epithelium resulting Th17 induction [10]. Notably, a diverse set of other intestinal bacteria with the ability to attach to the epithelium has been demonstrated to induce Th17 cell differentiation [11]. In addition to the classical antigen-dependent activation of T cells, bystander activation, i.e. the stimulation of unrelated T cells by cytokines during an immune response or inflammation, has emerged as potent activation mechanism of tissue-resident memory cells [12]. Specifically, protection mediated by SFB-induced Th17 cells during *C. rodentium* infection has been hypothesized to occur upon bystander activation. Recently we were able to show that specific members of the microbiota induced enhancement of IFN-γ production by CD4+ T cells, which contributed to resistance of mice against *Salmonella* infection [13]. While these results provide more evidence for the impact of the microbiota on CD4+ T cells in the intestine, it is not known yet, whether CD4+ T cells and specifically Th17 and Th22 cells primed by intestinal microbiota produce the same effector cytokines during antigen-induced and bystander activation *in vivo*.

Th17 and Th22 cells have been characterized in detail *in vitro*, however, technical difficulties such as the inability to determine gene expression profiles after ex-vivo restimulation and fixation of the cells have precluded a detailed *ex vivo* characterization of effector functions and diversity. Here, we have employed transgenic mouse lines, in which the expression of a fluorescent protein is under control of the endogenous promoter of a transcription factor or cytokines, i.e. FoxP3, IL-17A, and IL-22. These mouse lines allow the isolation of intact transcription factor or cytokine-expressing cells *ex vivo* based on their expression of individual or combinations of fluorescent proteins. Using these mouse lines, we characterized CD4+ T cell subsets induced either by the microbiota and as control activated during inflammation. Our novel data demonstrated that presence of SFB in the intestinal microbiota result in bystander activation of IL-17A and IL-22 producing CD4+ T cells upon *Salmonella* Typhimurium infection and can provide protection from colonization of the pathogen in intestinal tissue. Investigation of gene expression profiles by low-input RNAseq revealed distinct properties of these SFB modulated bystander activated T cell subsets compared to antigen-activated T cells including differential expression of IL-17F and IFN-γ. Notably, recent studies using multi-parameter flow cytometry have shown the presence of numerous tissue-specific Th cell subsets with distinct cytokine expression throughout the intestine and numerous other tissues in human body [14]. Hence, characterizing the diversity of CD4+ T cell responses and particularly Th17 and Th22 responses induced by the microbiota will be essential to understand the contribution of microbiota-induced memory T cells to host pathophysiology.

3.3 Experimental procedures

Mice: Wild type and all transgenic mice, IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP}, RORγt^{GFP} FoxP3^{RFP}, IL-17A^{GFP} IFN-$\gamma^{Katushka}$ FoxP3^{RFP} and IL-17A^{GFP} IL-22^{BFP}IFN-$\gamma^{Katushka}$ FoxP3^{RFP} reporter mice used in the study were on C57BL/6N background and have been rederived into SPF-1 microbiota by

embryo transfer and bred at the specific pathogen free animal facilities of Helmholtz Centre for Infection Research (HZI). SFB-monocolonized NOD/scid mice were bred in gnotobiotic isolators at the Medical University Hannover, Germany. Colonization with SFB was achieved by cohousing or oral gavage with intestinal content from SFB-monocolonized mice at least four weeks before infection experiments. Germ-free C57BL/6NTac mice were bred in isolators (Getinge) in the germ free facility of the HZI. All experiments were performed with 8-12 weeks old age-matched and gender-matched animals. Both male and female animals were used for every experiment to exclude influence of gender.

Colitis model induced by infection with *S.* Typhimurium: Naturally streptomycin-resistant wild-type strain *S. enterica* serovar Typhimurium SL1344 and isogenic mutant Δ*AroA* were used. *Salmonella* strains were grown overnight at 37°C in Luria-Bertani (LB) broth, with either 50µg/ml kanamycin or 100µg/ml streptomycin, then, diluted 1:100 in fresh medium, and subcultured for 4 hours. Bacteria were washed twice in ice-cold phosphate-buffered saline (PBS) and then used for infection experiments. Water and food were withdrawn for 4 hrs before mice were treated with 20 mg / mouse of streptomycin by oral gavage (o.g.). Afterwards, mice were supplied with water and food ad libitum. 20 hrs after streptomycin treatment, water and food were withdrawn again 4 hrs before the mice were infected with 10^5 CFU of *S.* Typhimurium and $3x10^6$ CFU of Δ*AroA* in 200 µl PBS. Drinking water ad libitum was supplied immediately and food 2 hrs post infection (p.i.). Mice were weighted every day and survival was monitored.

Analysis of bacterial loads in intestinal content and systemic organs: All mice were euthanized by asphyxiation with CO_2 and cervical dislocation at indicated time points. Intestinal tissues (small intestine, cecum, colon) and organs (mesenteric lymph nodes, spleen) were removed aseptically. To collect fecal content, organs were flushed with sterile PBS. Organs were opened longitudinally, cleaned thoroughly with sterile PBS and weighted. Organs and content were homogenized in sterile PBS using a Polytron

homogenizer (Kinemtatica). Dilutions of homogenized samples were plated on LB plates containing 50 µg/ml Kanamycin or 100µg/ml streptomycin and incubated overnight at 37°C to determine CFUs.

Analysis of SFB loads from content and tissue: Fresh stool samples of mice were collected and immediately stored at -20°C. DNA was extracted according to established protocols using a method combining mechanical disruption (bead-beating) and phenol/chloroform-based purification [15]. Briefly, sample was suspended in a solution containing 500µl of extraction buffer (200 mM Tris, 20mM EDTA, 200mM NaCl, pH 8.0), 200µl of 20% SDS, 500µl of phenol:chloroform:isoamyl alcohol (24:24:1) and 100µl of 0.1 mm zirconia/silica. Samples were homogenized twice with a bead beater (BioSpec) for 2 min. After precipitation of DNA, crude DNA extracts were resuspended in TE Buffer with 100µg/ml RNase and column purified to remove PCR inhibitors (BioBasic). Quantitative PCR was performed to detect total bacteria and SFB using specific primer sets: 16S (F: 5'-ACTCCTACGGGAGGCAGCAGT and R: 5'- ATTACCGCGGCTGCTGGC) and SFB (F: 5'- GACGCTGAGGCATGAGAGCAT and R: 5'-GACGGCACGGATTGTTATTCA).

Isolation of colonic lamina propria leukocytes (cLPL) and flow cytometry: To isolate cLPL, density gradient centrifugation using Percoll was done as previously described [16]. In brief, colons were collected during steady state and at d5 of DSS treatment. Fecal content was removed, tissues were opened longitudinally, washed with PBS and then shaken in HBSS containing 2 mM EDTA for 20 min at 37°C. Tissues were cut into small pieces and incubated with digestion solution (DMEM containing 1% fetal bovine serum (FBS), 0.25 mg/ml collagenase D, 0.5 U/ml dispase and 5 μg/ml DNase I) in a shaker for 20 min at 37°C. Digested tissues were filtered through 70uM cell strainer (Falcon) and DMEM + 5% FBS was added to inactivate enzymes. The last two steps were repeated until all tissue was digested. After centrifugation, cells were resuspended in 4 ml of 40% Percoll (GE Healthcare) and overlaid on 4 ml of 80% Percoll. Percoll gradient separation was

performed by centrifugation at 450 g for 25 min at 25°C. Cells in the interphase were collected and used as LPL. The collected cells were then suspended in staining buffer containing PBS, 1% FBS and 2 mM EDTA. The following antibodies were used: anti-CD45 (30-F11), anti-CD3 (17A2), anti-CD4 (RM4-5, GK1.5), anti-CD8a (53-6.7), anti-TCR$\gamma\delta$ (GL3), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CCR6 (29-2L17) (Biolegend) and anti-Vβ-14 (14-2) (BD Bioscience). To distinguish live dead cells AlexaFluor-350 NHS Ester (Life Technologies) was used. Flow cytometry analysis was performed using a BD LSR (BD Biosciences) and data were analyzed with FlowJo software (TreeStar Inc.).

RNA isolation and quantitative PCR: Tissues were preserved in RNAlater solution (Ambion) and subsequently homogenized in Trizol reagent (Invitrogen). One microgram of total RNA was used to generate cDNA by the protocol for first strand cDNA synthesis using RevertAid RT (Thermo Scientific). RealTime-PCR was performed using gene-specific primer sets (Sigma) of *Il17a* primer (F: 5'- ACGTTTCTCAGCAAACTTAC and R: 5'-CCCCTTTACACCTTCTTTTC); *Il17f* primer (F: 5'-ATACCCAGGAAGACATACTTAG and R: 5'- AGTCCCAACATCAACAGTAG); primer sets (Life Technologies) *Ifng* primer (Mm00801778_m1); *Il22* primer (Mm04203745_mH) and Kapa Sybr Fast or Probe qPCR kit (Kapa Biosystems) on a LightCycler 480 instrument (Roche). PCR conditions were 95^0C for 60 s, followed by 40 cycles of 95^0C for 3 s and 60^0C for 30 s. Data were analyzed using the the deltaCt method with *hprt* (F: CTGGTGAAAAGGACCTCTCG and R: TGAAGTACTCATTATAGTCAAGGGCA) serving as the reference housekeeping gene.

Low-input RNA sequencing: A minimum of 1000 cells was sorted and RNA was isolated using the RNAeasy plus micro Kit (Qiagen). RNA samples were used to synthesize and amplify cDNA (SmartSeq v4 3' DE Kit, Takara) followed by DNA library preparation for sequencing (Nextera XT, Illumina). This approach allowed us to multiplex up to 12 samples / HiSeq run. We

employed an analysis pipeline to determine differentially expressed genes. Briefly, raw reads were quality filtered using Trimmomatic and aligned to the mouse reference genome (mm10) using STAR. Normalization and differential expression were quantified using the DEseq2 package. Differential expressed gene networks were analyzed using the Consensus Path DB-mouse webserver.

Field emission scanning electron microscopy (FESEM): Gut samples were fixed with 5% formaldehyde and 2% glutaraldehyde in HEPES buffer (0.1 M HEPES, 10 mM CaCl2, 10 mM MgCl2 and 0.09 M sucrose, pH 6.9) overnight at 7°C, then washed twice with TE buffer (20 mM TRIS, 2 mM EDTA, pH 6.9) and gut content was squeezed out. Dehydration was achieved with a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 15 min for each step. Samples in the 100% acetone step were allowed to reach room temperature before another change in 100% acetone. Samples were then subjected to critical point drying with liquid CO2 (CPD 300, Leica, Wetzlar). Dried samples were covered with a gold-palladium film by sputter coating (SCD 500 Bal-Tec, Liechtenstein) before examination in a field emission scanning electron microscope Zeiss Merlin (Oberkochen) using the Everhart-Thornley SE-detector and the Inlens SE-detector in a 40:60 ratio with an acceleration voltage of 5 kV.

Statistical analyses: Statistical analysis was performed using GraphPad Prism program (GraphPad Software). Data are expressed as mean ± SEM. Differences were analyzed by Student's t test and ANOVA. *P* values indicated represent a non-parametric Mann-Whitney U test or Kruskal-Wallis test comparison between groups. *P* values ≤ 0.05 were considered as significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.4 Results

3.4.1 CD4+ T cells are the central source of in vivo IL-17A and IL-22 production upon enteric infection

The cytokines IL-17A and IL-22 play crucial roles during homeostasis and inflammation and are produced by various cell types during mucosal inflammation. To assess *in situ* production of these cytokines we took advantage of a novel IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} triple reporter mouse line (S. Huber, unpublished data). Specifically, we investigated the source of *in vivo* IL-17A and IL-22 production during steady state conditions as well as at early time points after *Salmonella* Typhimurium infection. Therefore, we infected streptomycin-pretreated IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} mice raised under conventional housing conditions with *S.* Typhimurium (*S.* Tm.) or left them untreated (steady state) and then isolated leukocytes from intestinal lamina propria (LPL) and lymphoid organs 20 hrs post infection (p.i.) (Figure 3.1A).

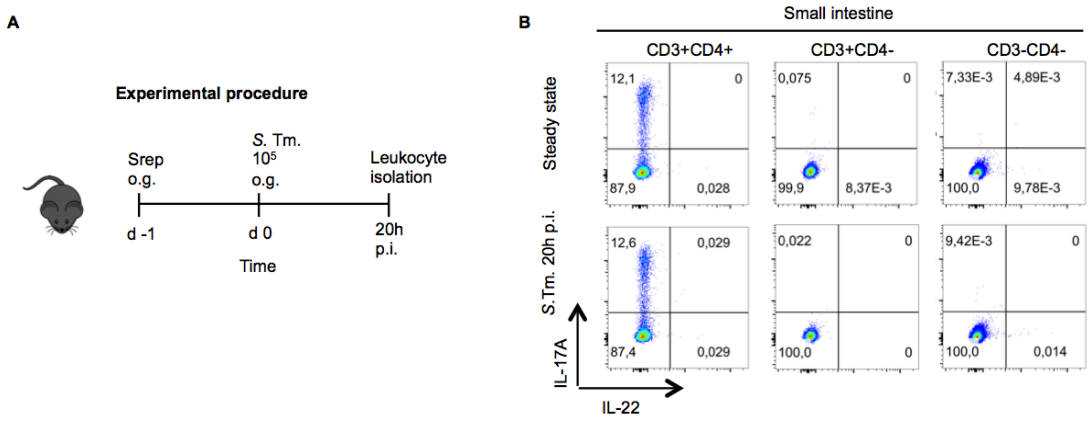

Figure 3.1 In-vivo IL-17A and IL-22 production in small intestine.

Experimental procedure for infection with *Salmonella enteria* serovar Typhimurium (*S.* Tm.), briefly Streptomycin-pretreated mice were orally infected with 10^5 of *S.* Tm. (A). Laminar propria leukocytes (LPL) were isolated from small intestine of non-infected (Steady state) and 20h after *S.*Tm. (*S.*Tm. 20h p.i.) infected IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} (conventionally raised) mice. Representative FACS plots are shown for IL-17A and IL-22 production from different cell subsets (B). Data represent n=4-6 mice/group from at least two independent experiments.

In vivo IL-17A production in mice during steady state was in small intestinal and cecal LPLs mainly derived from CD3+CD4+ T cells, likely reflecting the presence of SFB in our conventional mouse facility (Figure 3.1B, 3.2A). Notably, since *S*. Tm. induces inflammation mainly in the cecum *in vivo* IL-17A production from CD3+CD4+ small intestinal LPLs was not altered after infection with *S*. Tm (Figure 3.1B).

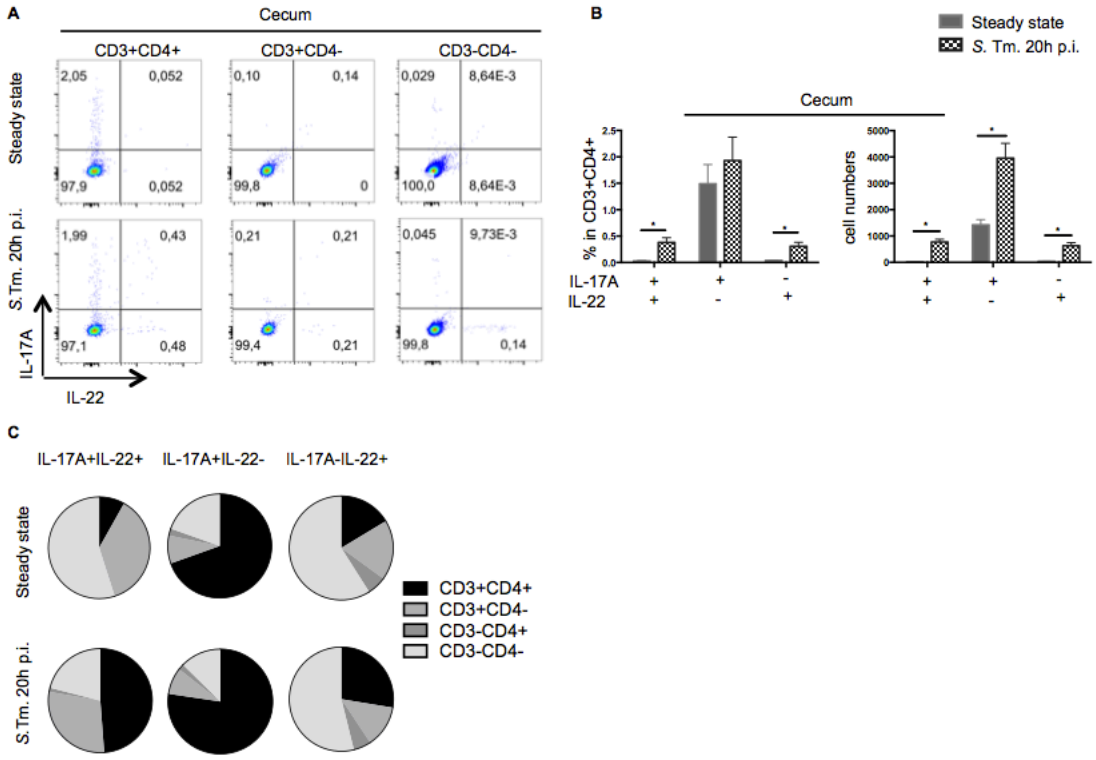

Figure 3.2 In-vivo IL-17A and IL-22 production in cecum.

LPL were isolated from cecum of non-infected (Steady state) and 20h after *S*.Tm. (*S*.Tm. 20h p.i.) infected IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} (conventionally raised) mice. Representative FACS plots are shown for IL-17A and IL-22 production from different cell subsets (A). Frequencies and absolute numbers of IL-17A and/or IL-22 production from CD3+CD4+ cells (B). Relative contribution of different leukocyte subsets for total production of IL-17A and IL-22 single or double cytokines (C). Data represent n=4-6 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

In contrast, direct *in situ* IL-22 production was detected mainly in cecal LPLs in infected mice, but absent under steady state conditions. Strikingly, CD3+CD4+ cells were the major *in vivo* source of IL-17A single and IL-17A/IL-22 double cytokine production in LPL from infected cecum (Figure 3.2A, 3.2B and 3.2C). Although CD3-CD4- cells were the main source of IL-22 single cytokine production in cecum, CD3+CD4+ production of IL-22 increased significantly after *Salmonella* infection (Figure 3.2C).

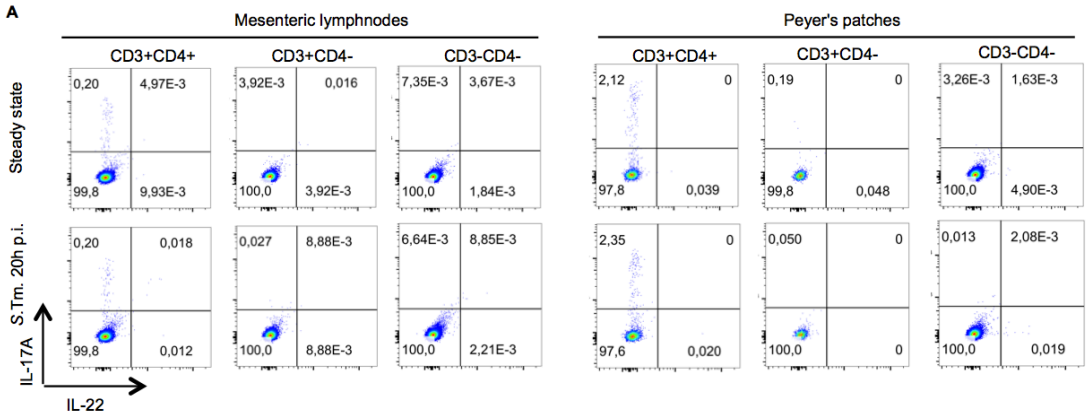

Figure 3.3 In-vivo IL-17A and IL-22 production in lymphoid organs.

Leukocytes were isolated from mesenteric lymphnodes and Peyer's patches of non-infected (Steady state) and 20h after *S*.Tm. (*S*.Tm. 20h p.i.) infected IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} (conventionally raised) mice. Representative FACS plots are shown for IL-17A and IL-22 production from different cell subsets (A). Data represent n=4-6 mice/group from at least two independent experiments.

Similarly, distinct lymphocytes subsets isolated from both mesenteric lymphnodes and Payer's patches demonstrated no *in vivo* IL-22 production (Figure 3.3A). Only IL-17A production was observed from CD3+CD4+ cell subsets from these lymphoid organs, however no major difference was detected between infected and non-infected groups (Figure 3.3A). Together, these data suggest that, CD4+ T cells are a significant source of early *in vivo* IL-17A and IL-22 responses in the intestinal mucosa after *Salmonella* infection.

3.4.2 *In vivo* early IL-17A/IL-22 response by CD4+ T cells upon *Salmonella* infection are dependent on SFB

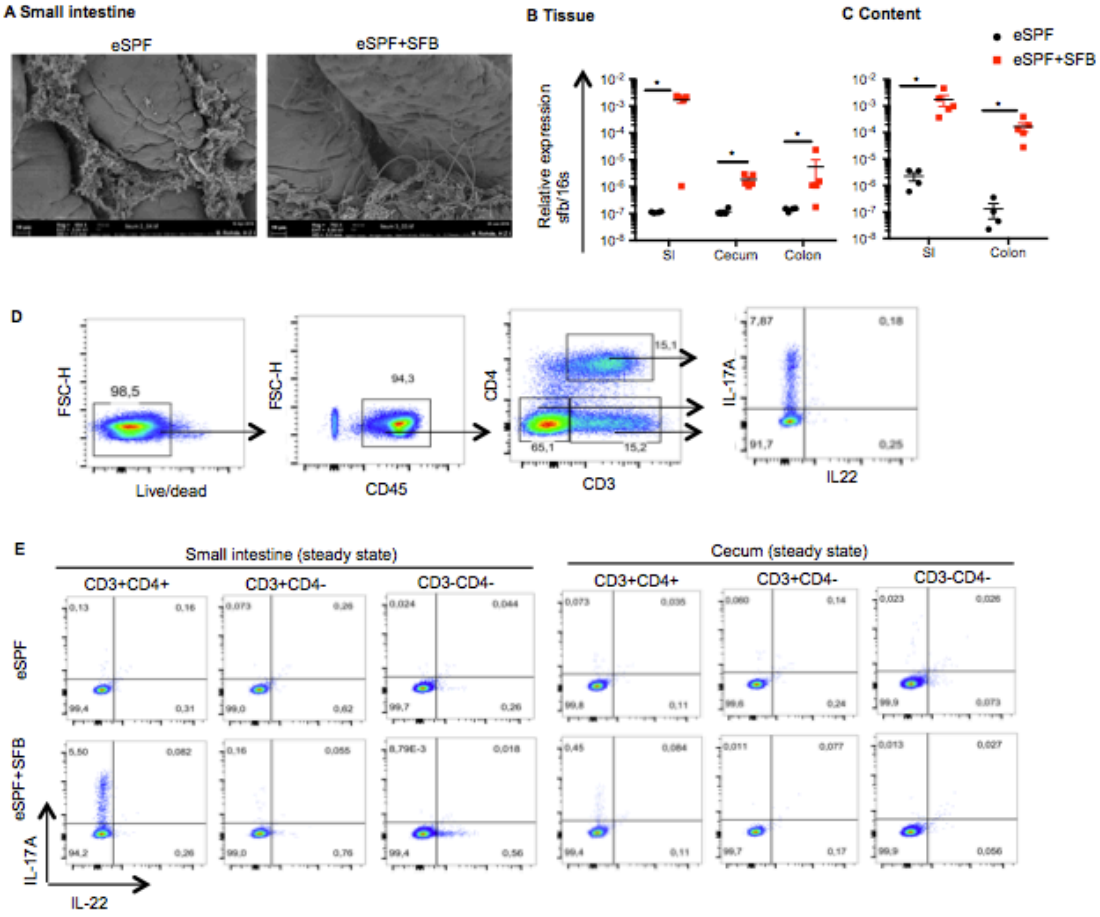

Figure 3.4 SFB colonization results in induction of only in-vivo IL-17A producing CD3+CD4+ cells in small intestine.

(A-C) eSPF IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} reporter mice mice were cohoused with SFB monocolonized mice. SFB colonization was detected by scanning electron microscopy of terminal ileum (A). SFB colonization in tissue (B) and intestinal content (C) was quantitatively assessed by qPCR.

(D-E) LPL were isolated from IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} mice harboring only eSPF or eSPF cohoused with SFB monocolonized mice (eSPF+SFB) during steady state. Gating strategy to characterize distinct LPL subsets is given (D). Representative FACS plots showing different subsets of LPLs gated on CD45+ cells releasing IL-17A and/or IL-22 in-vivo (E).

Data represent n=4-13 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Previous work has demonstrated that innate-like Th17 responses upon enteropathogenic infection are dependent on the intestinal microbiota [12]. To confirm this prior observation and further investigate the effect of the microbiota on *in vivo* cytokine production, we rederived IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} mice into an enhanced specific pathogen free (eSPF) microbiota condition lacking numerous known immunomodulatory components of microbiota, i.e. SFB, *Helicobacter spp*., norovirus and *Tritrichomonas spp*. that have been reported to influence CD4+ T cell responses. Specifically, SFB is known to induce Th17 response. To determine the influence of SFB on the *Salmonella* induced early CD4+ T cell response, we therefore cohoused our eSPF reporter mice with SFB monoassociated donor mice for 14 days. Scanning electron microscopy of the terminal ileum of SFB cohoused recipients demonstrated the presence of long segmented bacteria attached to the epithelium of SFB cohoused mice, which were absent in eSPF mice (Figure 3.4A). In addition, we characterized SFB colonization associated to the mucosa as well as in the lumen by performing SFB-specific qPCR. As expected SFB was enriched in the mucosa of the terminal ileum, but 100-fold decreased levels were detected associated with both the cecal and colonic mucosa of SFB-cohoused mice compared to SFB-deficient mice (Figure 3.4B). Notably, despite its enrichment in the mucosal fraction, SFB was also detected in the intestinal content in both SI and colon (Figure 3.4C). Next, we characterized different immune cells producing IL-17A/IL-22 from eSFP reporter mice in presence or absence of SFB during steady state (Figure 3.4D). In line with previous studies we observed significant increases in the frequency and number of IL-17A producing CD3+CD4+ T cells *in vivo* from small intestinal lamina propria of eSPF mice colonized with SFB (Figure 3.4E). Other distinct immune cell repertoire from small intestinal LPL and immune cells isolated from cecal LPL did not show any significant IL-17A/IL-22 induction at baseline (Figure 3.4E).

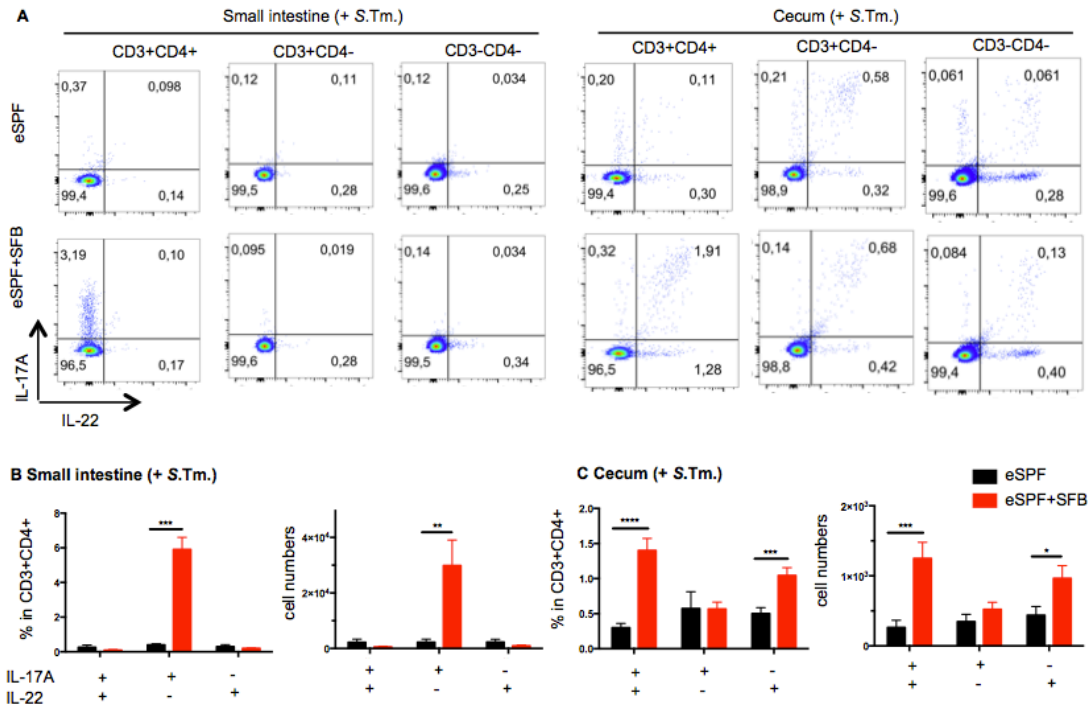

Figure 3.5 In-vivo IL-17A and IL-22 production after *Salmonella* infection is induced by SFB.

(A-C) LPL were isolated during 20h after *S*.Tm. infection from IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} mice harboring only eSPF or eSPF cohoused with SFB monocolonized mice (eSPF+SFB). Representative FACS plots showing different subsets of LPLs gated on CD45+ cells releasing IL-17A and/or IL-22 in-vivo (A). Percentage and absolute number of IL-17A and IL-22 single or double producing CD3+CD4+ T cells are shown from small intestine (B) and cecum (C). Data represent n=7-13 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

In contrast, infection with *Salmonella* pathogen induced a distinct cytokine milieu 20hrs post infection. Similar to conventionally housed mice, SFB colonized eSPF mice demonstrated significant increases in the frequencies and numbers of IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells in the cecal LPLs (Figure 3.5A, 3.5C). Interestingly, this increase of cytokine production was not observed in CD3+CD4- and CD3-CD4- cellular fractions of cecal LPLs (Figure 3.5A, 3.5C). Similar to steady state conditions the presence of SFB resulted in induction of only IL-17A production from CD3+CD4+ T cells in small intestinal LPL from *Salmonella* infected mice (Figure 3.5A, 3.5B). No additional cytokine production was observed in presence of SFB by distinct

subsets of small intestinal LPLs after *Salmonella* infection (Figure 3.5A, 3.5B). Notably, absolute numbers of these different cell populations supported the observation indicating SFB mediated increased absolute recruitment of these cells specifically IL-17A-IL-22+ and IL-17A+IL-22+ CD3+CD4+ T cells in the site of infection (Figure 3.5B, 3.5C).

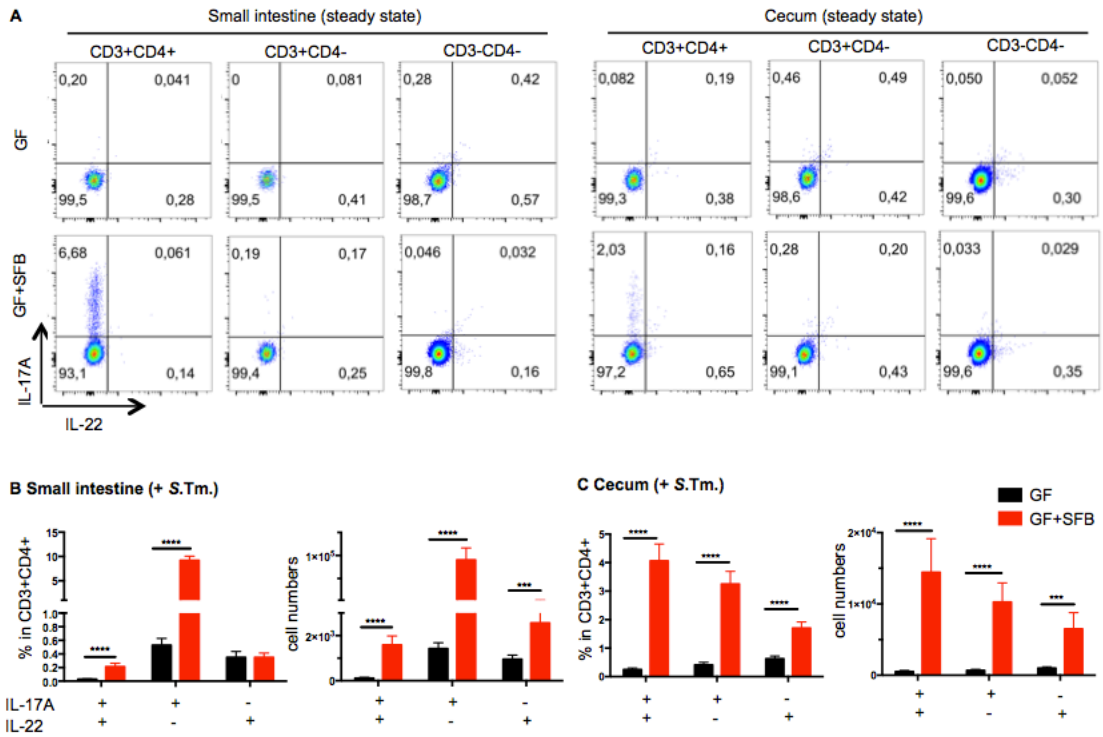

Figure 3.6 In-vivo IL-17A and IL-22 production in germ free (GF) mice.

LPL were isolated during steady state and 20h after *S.*Tm. infection from IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} GF mice or GF cohoused with SFB monocolonized mice (GF+SFB). Representative FACS plots showing different subsets of LPLs gated on CD45+ cells releasing IL-17A and/or IL-22 in-vivo during steady state (A). Percentage and absolute number of IL-17A and IL-22 single or double producing CD3+CD4+ T cells are shown from small intestine (B) and cecum (C). Data represent n=7-13 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

To exclude the effect of different food antigens and/or interaction of SFB with other members of the intestinal microbiota on our observations, we rederived IL-17A^{GFP} IL-22^{BFP} Foxp3^{RFP} mice into germ-free (GF) conditions. Thereby, we could cohouse GF reporter mice with monoassociated SFB mice to monitor whether SFB is sufficient to induce these distinct cytokine producing cells *in vivo*. Similar to SFB cohoused eSPF mice, monoassociation of GF mice with SFB also resulted in significant production of IL-17A from small intestinal CD3+CD4+ LPLs at baseline (Figure 3.6A). Interestingly, in contrast to eSFP mice, cecal LPLs isolated from GF mice during steady state demonstrated increase in IL-17A production from CD3+CD4+ cells only in presence of SFB (Figure 3.6A). During infection with *S*. Tm., in line with the observations from the eSPF mice, we detected increased frequencies and absolute numbers of IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells in cecal LPLs from *S.* Tm. infected SFB colonized GF mice (Figure 3.6C). Unlike eSPF mice, *S.* Tm. infection resulted in robust accumulation of additional IL-17A+IL-22- CD3+CD4+ T cells in cecal LPL and IL-17A-IL-22+ and IL-17A+IL-22+ CD3+CD4+ T cells also in the small intestinal LPLs of GF mice harboring SFB (Figure 3.6B, 3.6C). CD3+CD4- and CD3-CD4- cells did not show any difference on *in vivo* cytokine production in presence or absence of SFB in GF mice (data not shown). Taken together, these data demonstrate that early production of IL-17A and IL-22 by CD4+ T cells after *Salmonella* infection require SFB priming.

3.4.3 SFB-specific CD4+ T cells in the cecum obtain effector function of cytokine production upon *Salmonella* infection

Both SFB induced Th17 cells and innate like Th17 cells have been reported to show activated memory-like phenotype [12]. We characterized the activation and memory state of SFB-induced IL-17A and/or IL-22 producing CD3+CD4+ T cells based on the expression of specific markers including CCR6, CD44 and CD62L in the presence or absence of *Salmonella* infection (Figure 3.7A, 3.7B).

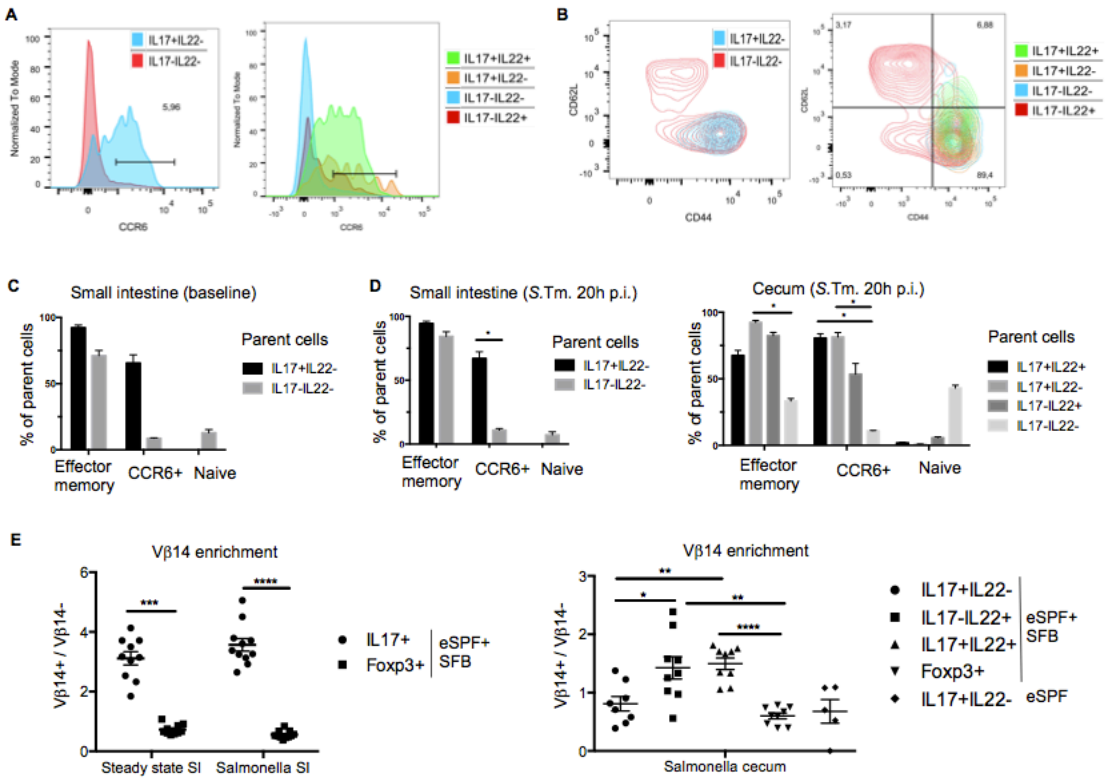

Figure 3.7 Characterization of SFB induced CD4+ T cells in eSFP mice using surface markers.

(A-E) LPL were isolated during steady state and 20h after *S*.Tm. infection from IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} mice harboring eSPF or eSPF+SFB. Representative plots showing expression of distinct surface markers from non-infected (left) and infected (right) animals of CCR6 (A) and CD44/CD62L (B) expression. Percentages of cytokine producing cells expressing distinct surface markers i.e. CD44^{hi}CD62L- (Effector memory), CCR6 or CD44^{lo}CD62L+ (naïve) in small intestine during baseline (C), in small intestine and cecum during *S*.Tm. infection (D). Enrichment of Vβ14 TCR in IL-17A and IL-22 producing CD4+ T cells compared to non-cytokine producing CD4+ T cells in small intestinal and cecal LPL (E). Data represent n=6-13 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a nonparametric Kruskal-Wallis test (D) and a unpaired Student's t test (C, D, E) *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

We observed that regardless of infection state in SFB colonized eSPF mice, small intestinal LPLs nearly 100% of IL-17A+IL-22- cells were effector memory (CD44+CD62L-) cells and around 70% of them expressed CCR6 when compared to IL-17A-IL-22- cells (Figure 3.7C, 3.7D). Strikingly, after *S.* Tm. infection, in cecal LPLs of SFB colonized eSPF mice IL-17A+IL-22+, IL-17A+IL-22- and IL-17A-IL-22+ cells demonstrated a similar effector memory like state as small intestinal IL-17A+IL-22- cells (Figure 3.7D). Notably, fewer IL-17A-IL-22+ CD4+ T cells expressed CCR6 compared to other cytokine releasing cell subsets in cecum. These findings were further corroborated by isolation and characterization of the LPLs from GF mice monocolonized with SFB (Figure 3.8A).

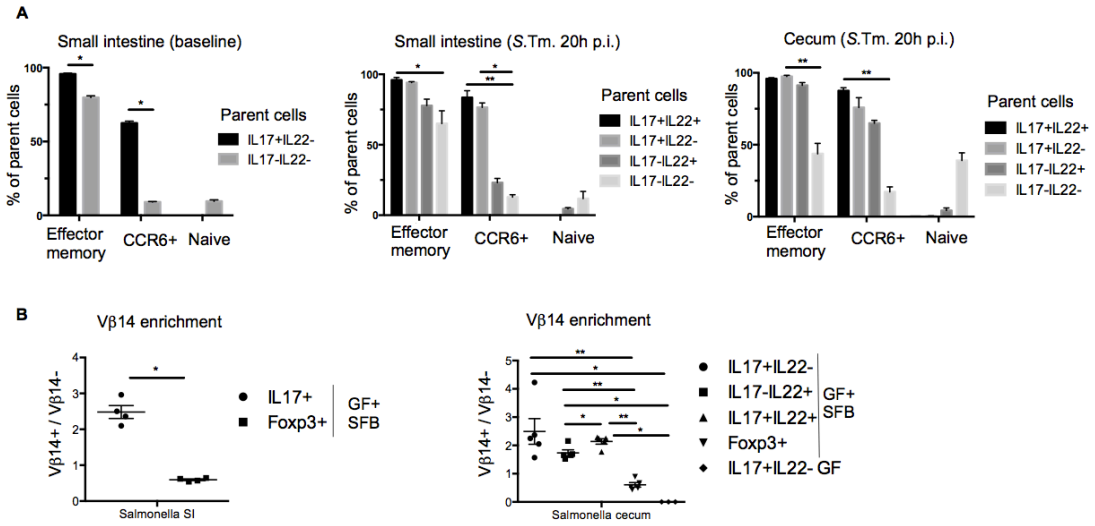

Figure 3.8 Characterization of SFB induced CD4+ T cells in GF mice using surface markers.

(A-B) LPL were isolated during steady state and 20h after *S.*Tm. infection from IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} mice harboring GF or GF+SFB. Percentages of cytokine producing cells expressing distinct surface markers i.e. CD44^{hi}CD62L- (Effector memory), CCR6 or CD44^{lo}CD62L+ (naïve) in small intestine during baseline, in small intestine and cecum during *S.*Tm. infection (A). Enrichment of Vβ14 TCR in IL-17A and IL-22 producing CD4+ T cells compared to non-cytokine producing CD4+ T cells in small intestinal and cecal LPL (B). Data represent n=5-13 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a nonparametric Kruskal-Wallis test (A) and a unpaired Student's t test (B) *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

It has been demonstrated that SFB-induced Th17 cells are specifically enriched in the Vβ14 chain of the TCR [17]. We therefore monitored Vβ14 TCR enrichment in distinct cytokine producing cells after *S.* Tm. infection. In line with the previous reports, small intestinal IL-17A+ (Th17) cells were significantly enriched with Vβ14 TCRs compared to Foxp3+ CD4+ T cells (Figure 3.7E). In cecal LPLs from SFB colonized eSPF mice infected with *S.* Tm., significant increase in the Vβ14 TCR enrichment was observed specifically in IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells compared to IL-17A+IL-22- and Foxp3+ CD4+ T cells (Figure 3.7E). We performed similar experiments in SFB monocolonized GF mice and could show increase in Vβ14 TCR enrichment in different CD4+ T cell subsets similar to that observed in eSPF+SFB mice (Figure 3.8B). Unlike in eSPF+SFB mice increased Vβ14 TCR enrichment was also observed in IL-17A+IL-22- CD4+ T cell subset in cecal LPLs after *S.* Tm. infection (Figure 3.8B).

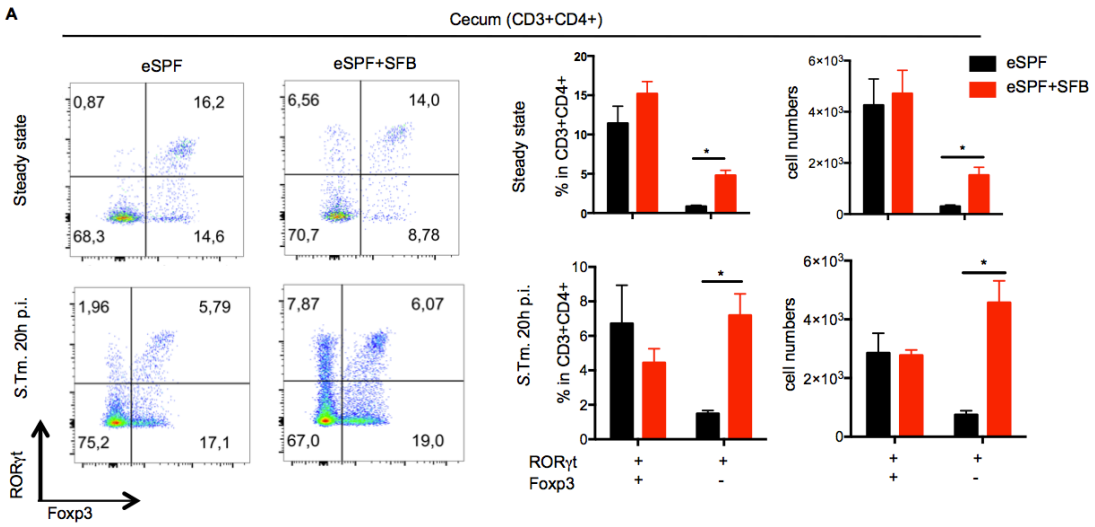

Figure 3.9 SFB modulation of in-vivo CD4+ T cells expressing distinct transcription factors.

LPL were isolated during steady state and 20h after *S.*Tm. infection from cecal tissues of RORγt^{GFP} FoxP3^{RFP} mice harboring eSPF or eSPF+SFB. Representative FACS plots and percentages and absolute numbers of CD3+CD4+ cells expressing RORγt and Foxp3. Data represent n=6-13 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Transcription factor RORγt plays critical role for differentiation of Th17 cells. We postulated that SFB primed CD4+ T cells already seed the cecal lamina propria and obtain effector function of IL-17A and/or IL-22 secretion after *Salmonella* infection. In order to investigate the existence of *in vivo* precursor for distinct cytokine producing CD4+ T cells we obtained previously described RORγt^{GFP}FoxP3^{RFP} reporter mice [18] and rederived them under eSPF microbiota condition. Indeed, analysis of CD3+CD4+ cecal LPL revealed significantly increased frequencies and numbers of RORγt+ cells in SFB colonized eSPF mice (Figure 3.9A). Although *Salmonella* infection did largely not affect the frequencies of RORγt+ cells, the absolute numbers were much higher in mice harboring SFB indicating enhanced differentiation of these cells (Figure 3.9A). Previous works demonstrated influence of microbiota specifically SFB on RORγt+Foxp3+ and Foxp3+ CD4+ T cells [19,20]. However, in our eSPF facility, SFB colonization does not seem to affect these cells (Figure 3.9A). These results demonstrate that SFB colonization results in the presence of resting RORγt+CD4+ T cells in cecal lamina propria and during early infection with *Salmonella* pathogen SFB-specific CD4+ T cells differentiate to produce IL-17A and/or IL-22.

3.4.4 Bystander activation of SFB-dependent IL-17A and/or IL-22+ CD4+ T cells have distinct gene-expression profile

Next we wanted to characterize the detailed transcriptomic profile of distinct *in vivo* cytokine producing cells comparing antigen-specific and bystander activation by sorting these cells based on their cytokine expression directly from *in vivo*. From SFB colonized eSPF mice, we FACS sorted 5 distinct population of CD3+CD4+ T cells: IL-17A+IL-22- cells from small intestinal LPLs of uninfected mice as well as IL-17A+IL-22- T cells from small intestinal LPLs, IL-17A+IL-22-, IL-17A-IL-22+ and IL-17A+IL-22+ cells from cecal LPLs of *S.* Tm. infected mice (Figure 3.10A).

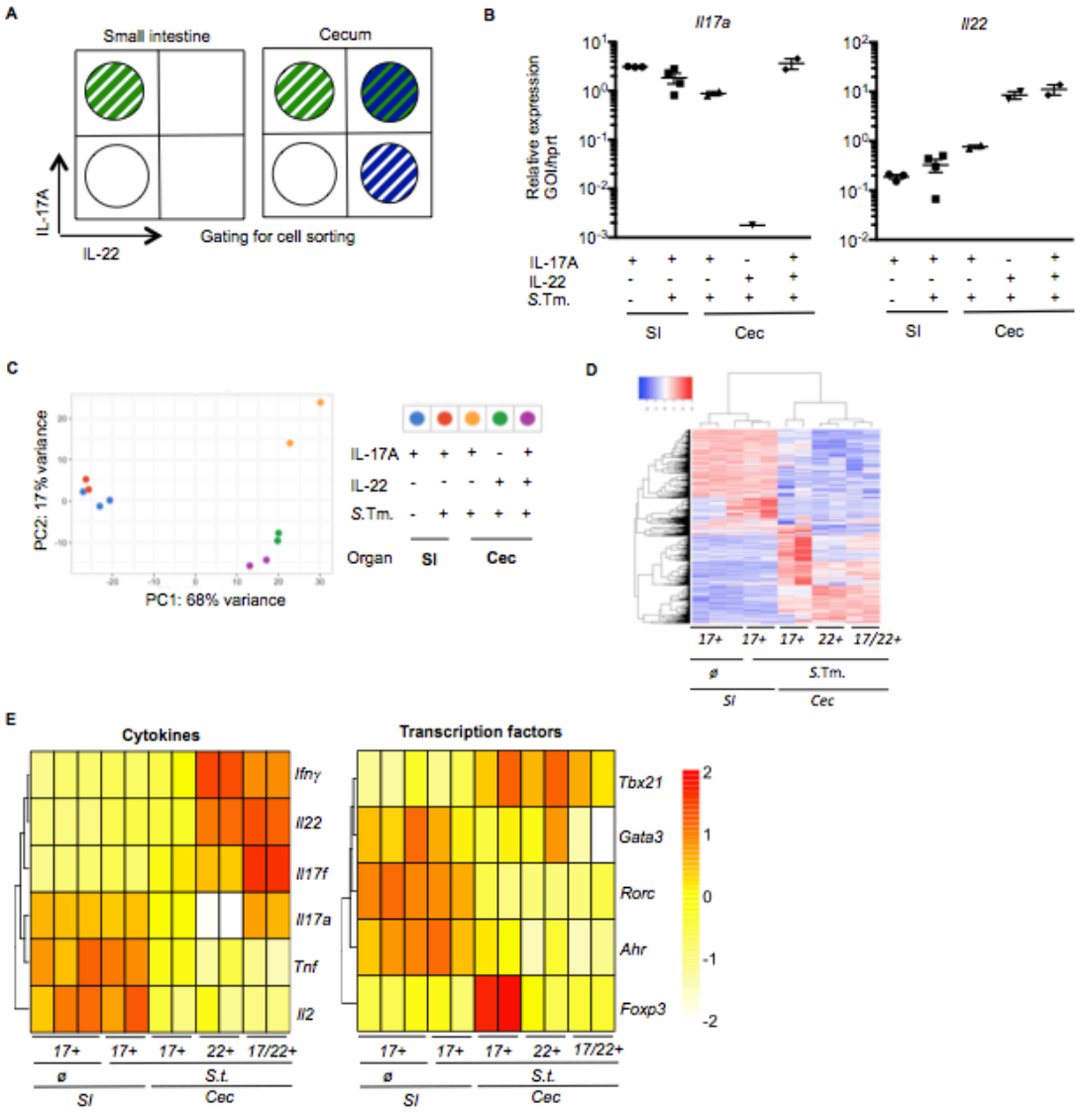

Figure 3.10 Characterization of SFB induced CD4+ T cells in eSFP mice using RNA-seq.

Small intestinal and cecal CD3+CD4+ LPL were FACS sorted based on their expression of IL-17A and/or IL-22 (A). Real time qPCR was performed to validate and confirm the purity of FACS sorted cells using specific primers to detect *Il17a*, *Il22* (B).

(C-E) RNA-seq analysis of FACS sorted cells was performed. Analysis of β-diversity (PCoA) of distinct cell subsets based on their transcriptome profile (C). Heatmap shows quantification of RNA reads for normalized all differentially expressed gene sets (D) and differential expression of specific cytokines and transcription factors (C) among the groups. Data represent n=2-4 sample/group.

Minimum duplicates of 1000 cells with distinct properties were sorted and low-input RNAseq was performed according to the protocol describe in method section. The purity of different subsets of FACS sorted cells was confirmed using specific qPCR for *Il17a* and *Il22* gene expression (Figure 3.10B). Interestingly, analysis of β-diversity of transcriptome data using PCoA revealed that IL-17A+IL-22- CD4+ T cells from small intestine clustered together regardless of infection state (Figure 3.10C). Analysis of global gene expression of these two groups also demonstrated similar gene expression signatures (Figure 3.10D). Strikingly, IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells from infected cecal LPLs clustered together and demonstrated distinct gene expression profile from that of small intestinal IL-17A+IL-22- cells (Figure 3.10C, 3.10D). IL-17A+IL-22- CD4+ T cells from cecal tissue demonstrated unique properties of completely separate clustering in PCoA analysis and unique gene expression signature (Figure 3.10C, 3.10D). Next, we focused on identifying specific differences in terms of distinct cytokine and transcription factor expression among the groups. To our surprise, we observed alterations in expression of *Ifnγ* and *Il17f*. In contrast to IL-17A+IL-22- cells from all conditions IL-17A-IL-22+ and IL-17A+IL-22+ cells expressed significantly higher *Ifnγ* and *Il17f* (Figure 3.10E). Strikingly, *Il17f* expression was highest in IL-17A+IL-22+ CD4+ T cells (Figure 3.10E). A small increase in *Tnf* and *Il2* expression was observed in small intestinal IL-17A+IL-22- cells (Figure 3.10E). Notably, we did not detect any major differences in expression of different transcription factors among these groups. Small intestinal T cells expressed somewhat higher *Gata3*, *Rorc* and *Ahr*, whereas cytokine-producing cells derived from cecum showed higher *Tbx21* expression (Figure 3.10E). Interestingly, strong *Foxp3* expression was monitored from cecal IL-17A+IL-22- cells suggesting that this fraction contains regulatory T cells (Figure 3.10E). To confirm our observation regarding discrepancies on *Ifnγ* and *Il17f* expression, we performed gene-specific qPCR on *in vivo* FACS sorted cells. In line with the transcriptome data, we observed high expression of *Ifnγ* and *Il17f* in IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells (Figure 3.11A).

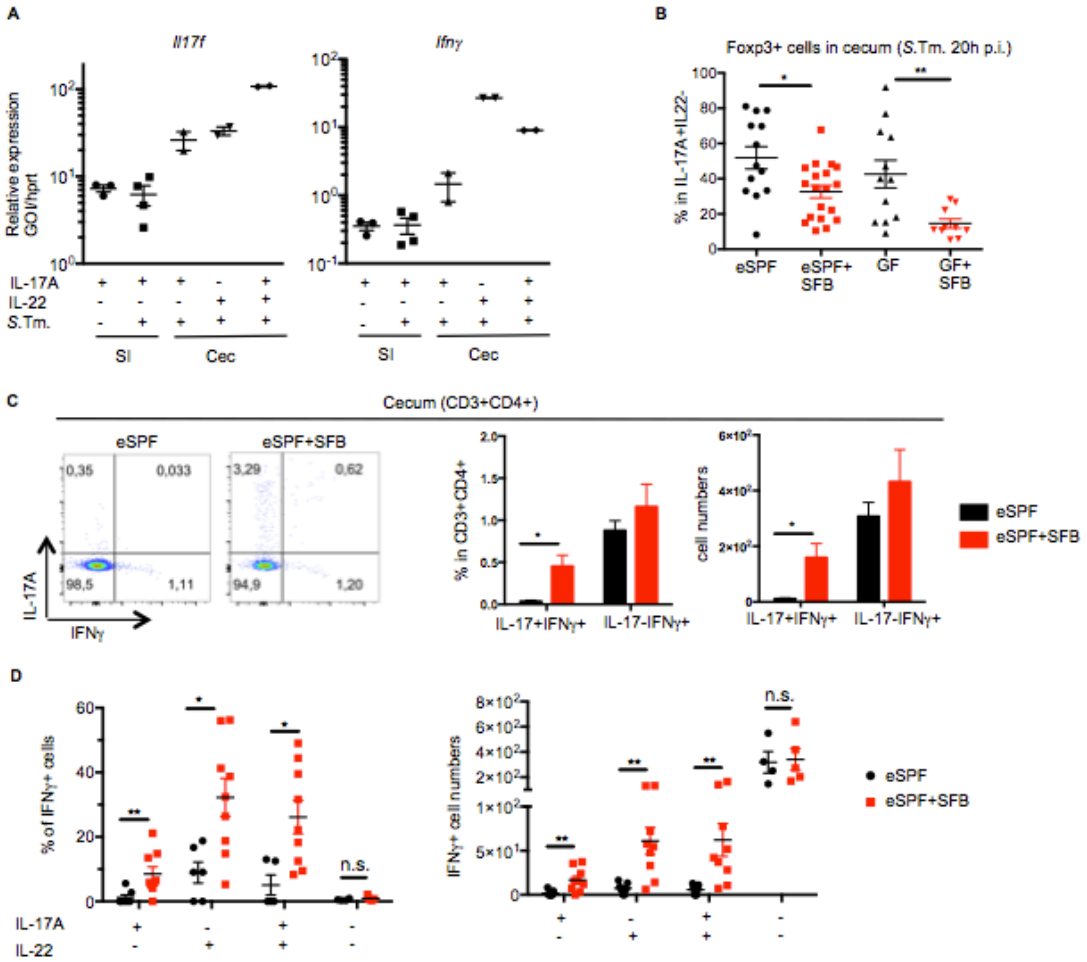

Figure 3.11 Validation of RNA-seq results in cytokine knock-in reporter mice.

Real time qPCR was performed to validate *Il17f* and *Ifnγ* expression in FACS sorted cells from Figure 10A (A).

LPL were isolated from cecum during 20h after *S.*Tm. infection from IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} eSPF and eSPF+SFB or GF and GF+SFB mice. Percentage of CD3+CD4+IL-17A+IL-22- cells expressing Foxp3 was measured (B).

LPL were isolated from cecum of IL-17A^{GFP} IFN-γ^{Katushka} FoxP3^{RFP} mice harboring eSPF or eSPF+SFB. Representative FACS plots and percentages and absolute numbers of CD3+CD4+ cells producing IL-17A and/or IFNγ (C).

LPL were isolated from cecum of IL-17A^{GFP} IL-22^{BFP}IFN-γ^{Katushka} FoxP3^{RFP} mice harboring eSPF or eSPF+SFB. Percentage and numbers of different IL-17A and/or IL-22 producing CD3+CD4+ cells demonstrating in-vivo IFNγ expression (D).

Data represent n=2-20 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

SFB modulation of IFNγ producing CD4+ T cells (Th1) from the previous works was inconsistent; from no influence of SFB to induce Th1 to SFB modulated strong Th1 response [9,21] . Using cytokine knock-in reporter mice we aimed to monitor the effect of SFB on *in situ* IFNγ producing cells. Therefore we obtained previously described IL-17A^{GFP} IFN-γ^{Katushka} FoxP3^{RFP} mice [22] and rederived them on eSPF microbiota background. Indeed, during early stage of *Salmonella* infection IL-17A+IFNγ+ CD4+ T cells from cecal LPLs were significantly increased in SFB colonized eSPF mice (Figure 3.11C) suggesting SFB modulation of a unique subset of CD4+ T cells producing multiple cytokines i.e. IL-22, IL-17A, IFNγ in infected tissues. There were no significant differences in CD4+ T cells producing only IFNγ in presence or absence of SFB (Figure 3.11C). However, to confirm generation of *in vivo* multiple cytokine producing cells modulated by SFB we crossed our two reporter mouse lines to create novel quadriple IL-17A^{GFP} IL-22^{BFP} IFNγ^{Katushka} Foxp3^{RFP} reporter mice. Isolation of LPL from *Salmonella* infected cecal tissue revealed significant increase in IFNγ production by IL-17A and IL-22 both single and double producing CD4+ T cells in SFB colonized eSPF mice (Figure 3.11D). Specifically, similar to the findings from transcriptome analysis, the highest production of IFNγ were from IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells (Figure 3.11D). Since transcriptome analysis revealed significant *Foxp3* expression by IL-17A+IL-22- CD4+ T cells in *Salmonella* infected cecal LPLs we measured the Foxp3 expression in our *in vivo* isolated LPLs from cytokine reporter mice. Indeed, after *Salmonella* infection only IL17+IL-22- CD4+ T cells present in cecum demonstrated increased *Foxp3* expression (data not shown). Interestingly, IL17+IL-22- CD4+ T cells from SFB deficient mice had significantly higher frequencies of Foxp3+ cells compared to that from SFB colonized both eSPF and GF mice (Figure 3.11B). Together, these data illustrate distinct properties of SFB-induced bystander activated cells and suggest that innate CD4+ T cells modulated by SFB after enteropathogen infections are able to play unique function of multiple cytokine secretion to combat the infection.

3.4.5 SFB colonization reduces growth of *Salmonella* in local tissue

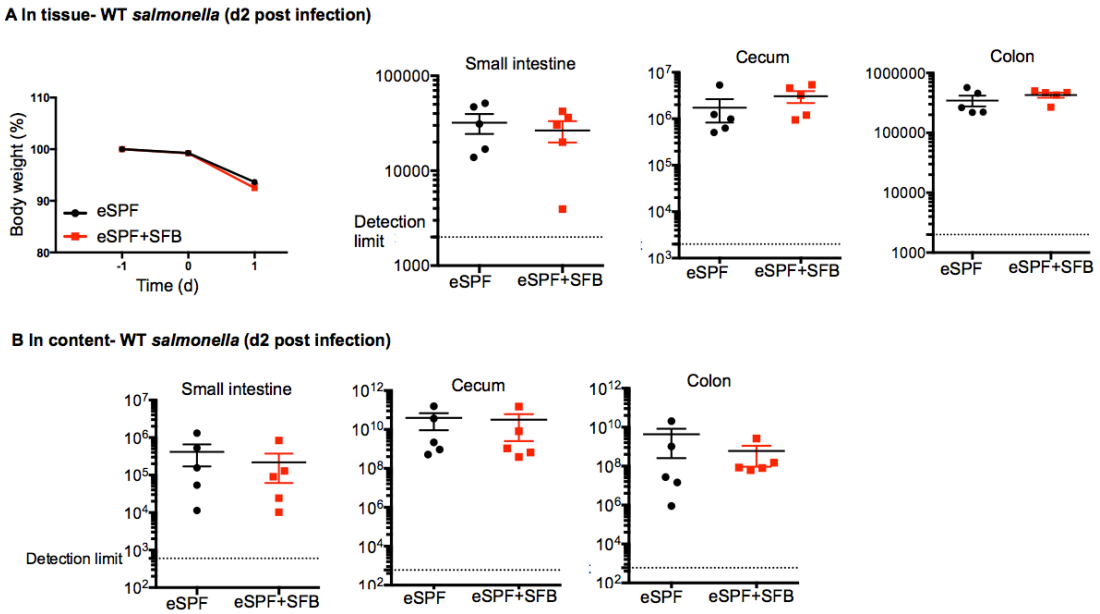

Figure 3.12 Colonization of wild type (WT) *Salmonella* in intestinal tissues and content in eSFP mice.

eSPF and eSPF+SFB mice were infected with *S.* Tm. wild-type. Body weight of infected mice is shown (A). WT *Salmonella* infected mice were sacrificed 2 days p.i. and *S.* Tm. CFUs were determined in tissue (A) and content (B) of small intestine, cecum and colon. Data represent n=5-8 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

SFB has been demonstrated to enhance resistance against *C. rodentium* infection [9], however, whether it regulates susceptibility to *Salmonella* colonization in the lumen or tissue invasion is not known. To test whether SFB colonization affects *Salmonella* infection, we employed the antibiotic-induced gastroenteritis model and infected eSPF and eSPF+SFB mice with WT *Salmonella*. Overall body weight loss after *Salmonella* infection did not differ in presence or absence of SFB (Figure 3.12A). Hence, we wanted to investigate whether presence of SFB in the intestinal microbial community can provide any colonization resistance against *Salmonella* growth and invasion into intestinal content and tissue. We detected similar number of *Salmonella* CFUs growing in the gastrointestinal tissue and lumen of both SFB colonized

and control eSPF mice 2 days post infection (Figure 3.12A, 3.12B). This demonstrates that SFB-induced alterations in the immune system are not able to significantly restrict WT *Salmonella.*

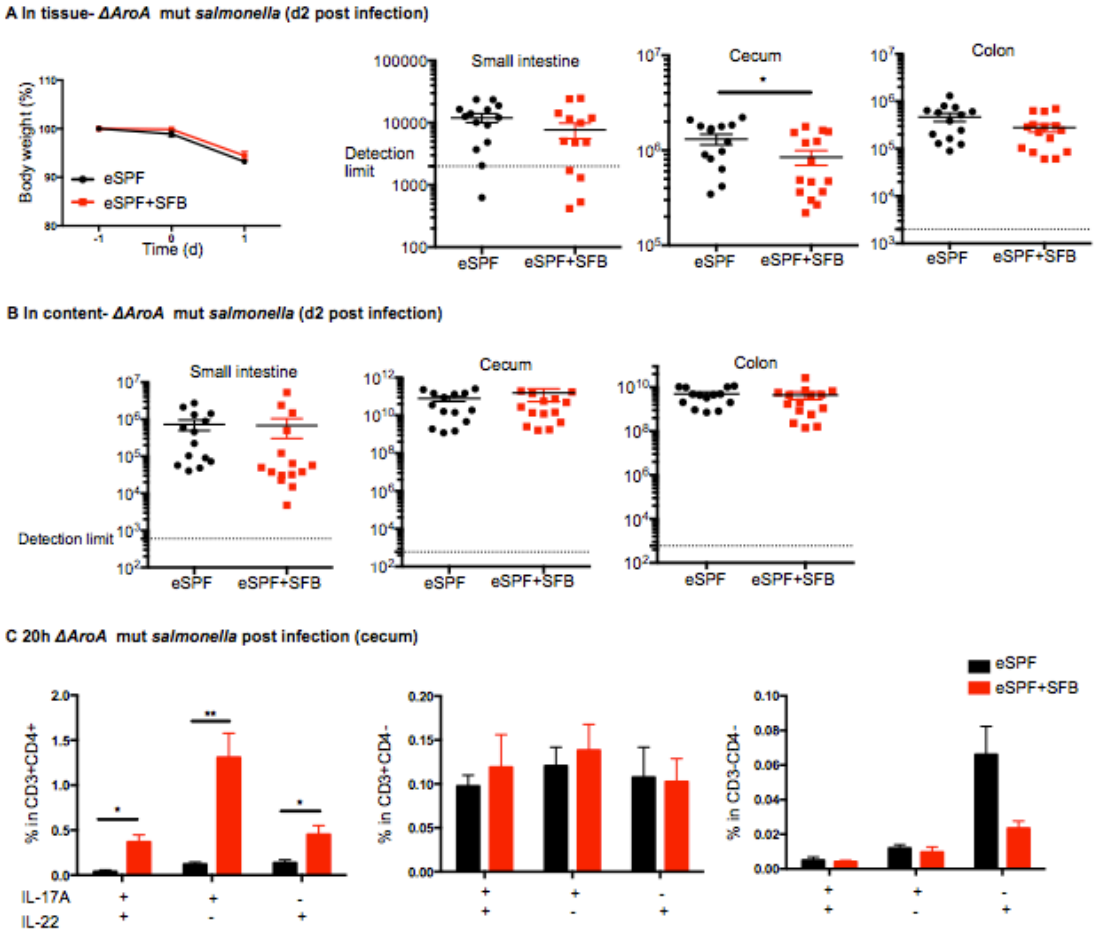

Figure 3.13 Colonization of Δ*AroA Salmonella* mutant in intestinal tissues and content in eSPF mice.

eSPF and eSPF+SFB mice were infected with *S.* Tm. Δ*AroA* mutant. Body weight of infected mice is shown (A). Δ*AroA Salmonella* infected mice were sacrificed 2 days p.i. and *S.* Tm. CFUs were determined in tissue (A) and content (B) of small intestine, cecum and colon. LPL were isolated 20h after Δ*AroA S.*Tm. infection from cecum of IL-17A^{GFP} IL-22^{BFP} FoxP3^{RFP} mice harboring eSPF or eSPF+SFB. Percentages of IL-17A and/or IL-22 production of distinct cell subsets are shown (C). Data represent n=6-16 mice/group as mean ± SEM from at least two independent experiments. P values indicated represent a unpaired Student's t test *p < 0,05; **p < 0,01; ***p< 0,001; ****p< 0,0001.

Notably, *Salmonella* can expand in the tissues of infected C57Bl/6 mice because these mice carry a spontaneous mutation in the Nramp1 gene [23], which impairs immune control significantly. Hence, we hypothesized that an attenuated strain of *Salmonella* with a decreased ability for systemic infection but still being able to elicit local infection might be affected by the presence of SFB-induced immune responses. Therefore, we infected eSPF and SFB colonized eSPF mice with a *S.* Tm strain lacking the aroA gene (Δa*roA)* and subsequently monitored pathogen colonization [24]. Similar to that of WT *S.* Tm*.,* infection with Δa*roA S.* Tm*.* also did not demonstrate any difference in body weight loss in presence or absence of SFB (Figure 3.13A). Strikingly, despite of similar colonization in the intestinal content, presence of SFB resulted in reduced colonization of the *Salmonella* mutant in the cecal tissue 2 days post infection (Figure 3.13A, 3.13B). Next, we wanted to confirm whether SFB-induced immunomodulatory effects were retained after infection with mutant *S.* Tm. Isolation of LPL from Δa*roA* mutant *S.* Tm*.* infected mice revealed comparable SFB-dependent innate-like cytokine responses from CD3+CD4+ T cells as in WT *S.* Tm. infected mice. Specifically, significant increase in both IL-17A and IL-22 production by CD3+CD4+ T cells from cecal tissue of SFB colonized mice was observed (Figure 3.13C). Finally, this data demonstrated that, SFB modulated bystander activation of CD4+ T cells is associated with local protection from pathogen colonization during *S.* Tm. infection.

3.5 Discussion

Understanding the contribution of specific members of the microbiota on immune homeostasis and inflammation has emerged as central question in many physiological and inflammatory processes including mucosal infections. In this study we demonstrate that a specific member of intestinal microbiota, SFB, can induce differentiation of Th17 and Th22 subsets, which upon infection induce proinflammatory and anti-microbial cytokines after bystander activation. Furthermore, we identified that the presence of bystander activated SFB-specific CD4+ T cells is associated with decreased *Salmonella* invasion into intestinal tissue.

In the GI tract, IL-17A and IL-22 have shown to contribute to immune protection against *Helicobacter pylori*, *Citrobacter rodentium* and *S.* Typhimurium [25–29]. These cytokines are produced by distinct subsets including Th17 and Th22 cells. Several studies have provided strong evidence of plasticity for these cell lineages both *in vivo* and *in vitro* depending on types of inflammation and culture conditions respectively [30–32]. Such plasticity of acquiring functional characteristics of other cell subsets i.e. secretion of non-canonical cytokine like IFNγ or IL-10 may play crucial role during specific inflammatory disease conditions. Therefore it is important to understand *in vivo* functional properties of these cell subsets under specific inflammatory condition. In the GI tract commensal microbiota shapes the intestinal immune system by regulating T helper (Th) cell lineage differentiation. For example, *Bacteroides fragilis* monocolonization not only establishes proper systemic Th1/Th2 balance, but also can induce regulatory T (Treg) cell differentiation in the gut [33,34]. In addition, SFB facilitate the development of Th17 cells in the small intestine or a mixture of 17 strains within clusters IV, XIVa, and XVIII of Clostridiales isolated from human feces can also induce the differentiation and expansion of Treg cells in the colon [35]. Recent studies have also demonstrated an effect of the gut microbiota on CD4 T cell during intestinal inflammation [12]. However, it is not clear whether antigen-specific classical CD4+ T cells are distinct from bystander-activated cells *in vivo* and which

specific member of the intestinal microbiota is responsible to induce such bystander activation of immune cells.

To investigate the diversity of intestinal CD4+ T cell responses directly from *in situ* using gene expression analysis have several caveats. First, subsets are largely distinguished by expression of transcription factors and cytokine expression. These can be assessed using antibodies and flow cytometry, but require fixation of the cell precluding the isolation of RNA. To allow isolation of cytokine-producing cells, so-called capture assays have been developed but these require restimulation of the cells *ex vivo*, which impacts their gene expression profiles. Finally, these cells are typically present in relatively low numbers complicating their analysis. To overcome these limitations, several groups have developed transgenic mouse lines, in which the expression of a fluorescent protein is under control of the endogenous promoter of a transcription factor or cytokine. These mouse lines allow the isolation of transcription factor or cytokine-positive cell ex vivo based on the expression of individual or combinations of fluorescent proteins [36]. In our study, we have employed two lines of mice allowing i) the identification of Foxp3 and Rorγt expressing cells ($Ror\gamma t^{GFP}Foxp3^{RFP}$) and ii) the identification of Foxp3, IL-17A, IL-22 and IFN-γ expressing cells ($IL\text{-}17A^{GFP}$ $IL\text{-}22^{BFP}$ $Foxp3^{RFP}$; $IL\text{-}17A^{GFP}$ $IFN\text{-}\gamma^{Katuska}$ $Foxp3^{RFP}$).

Using our novel transgenic reporter mice we could demonstrate that at early stage of Streptomycin-pretreated *S.* Typhimurium infected mice memory CD4+ T cells are the major source of IL-17A and IL-22 cytokine production. In line with previous work, we could observe an increase in the number of these innate-like types of IL-17A+IL-22- and IL-17A+IL-22+ CD4+ T cells in cecal LPL from infected mice [12]. Moreover, we also observed a significant increase in the numbers and frequencies in IL-17A-IL-22+ CD4+ T cells. However, in steady state condition our finding contrasted previous report of IL-22 reporter mice since we did not detect any IL-22 production from intestinal LPLs from non-infected mice [37]. This may reflect differences in microbiota and diet composition in animal colonies, since these factors have been demonstrated to modulate IL-22 production. Notably, IL-22 is not detectable in the colonic mucosa of healthy human subjects; however, IL-22 expression is readily

detectable from CD4+ T cells in the colonic mucosa of IBD patients [38]. Studies have demonstrated that the frequency of IL-22-producing cells is increased in UC patients as well as CD patients, indicating a possible pro-inflammatory role in etiology of IBD or an attempt of the cells to contribute to tissue repair [38,39]. To investigate the involvement of specific microbiota modulating such innate-like CD4+ T cells we rederived our reporter mice into an eSPF barrier lacking many known potential pathobionts i.e. SFB, *Helicobacter spp.*, Prevotellaceae, murine Norovirus, and murine protozoa that have previously been reported to modulate host adaptive immunity. Strikingly, mice from this eSPF barrier completely lost their ability to induce innate IL-17A and IL-22 producing CD4+ T cells upon *Salmonella* infection and cytokine production ability was successfully restored upon SFB colonization. We could also demonstrate that at early stages of *Salmonella* infection SFB can also induce CD4+ T cells producing only IL-22, which to our knowledge is the first report to observe microbiota modulation of Th22 cells. Unlike earlier studies that relied on *ex vivo* restimulation SFB colonization did not show any *in vivo* IL-22 producing Th17 cells at baseline, rather only classical IL-17A producing Th17 were observed in small intestinal LPLs. In line with previous findings, transcription factor knock-in reporter mice ($Ror\gamma t^{GFP}Foxp3^{RFP}$) showed ROR$\gamma$t+ Th17 cells were already present at baseline in SFB colonized cecal LPLs. Moreover, specificity of these innate IL-17A and IL-22 producing CD4+ T cells to SFB was confirmed since these cells expressed significantly higher Vβ14 TCR that is previously described as SFB-specific TCR [17]. Together these findings suggest that *in vivo* SFB is not only essential to induce steady-state Th17 cells in the small intestine, but also provide signals resulting in the development of CD4+ T cell subsets characterized by the ability to produce IL-22 and IL-17A cells rapidly after infection in the intestine.

To further characterize SFB-induced innate-like CD4+ T cells we employed transcriptional profiling of these distinct cell subsets directly isolated from intestinal LPLs based on their cytokine secretion. Strikingly, IL-17A+IL-22+ CD4+ T cells isolated from *S*. Tm infected mice demonstrated distinct gene expression profiles compared to Th17 cells isolated from the SI of SFB colonized mice at baseline and clustered more closely to IL-22 only producing

CD4+ T cells. Strikingly, these differences were mainly due to significant increased expression of IFNγ and IL-17F by IL-17A-IL-22+ and IL-17A+IL-22+ CD4+ T cells compared to Th17 cells. Increases in IFNγ expression might benefit the host via diverse mechanisms such as controlling pathogen loads in the intestinal tissue and regulating mucin release by goblet cells or other so far unknown effects [40–42]. Previous reports regarding the role of SFB to induce IFNγ producing CD4+ T (Th1) cells were contradictory demonstrating no Th1 induction to significant Th1 induction in presence of SFB [9,21]. Using different cytokine reporter mice we demonstrated a direct influence of SFB on IFNγ secretion from only IL-17A and/or IL-22 producing CD4+ T cells, but not from IL-17A-IL-22- CD4+ T (classical Th1) cells. Analysis of transcription factors revealed only a minor increase in *Tbx21* expression in these innate like CD4+ cells compared to classical Th17 cells. Detailed studies regarding the transcriptional regulation of these specific Th cell subsets are still necessary. After oral infection with wild type *S*. Tm. SFB colonization did not enhance pathogen clearance. However, increased numbers of multifunctional innate like CD4+ T cells induced by SFB significantly enhanced clearance of Δ*AroA* mutant of *S.* Tm from local tissue. This suggests SFB induced immunomodulatory effects in the GI tract are only able to provide protection from enteropathogen invasion locally. Further studies are needed to observe whether co-colonization of SFB with some other intestinal microbiota having immunomodulatory effects may synergize the protective effects against systemic enteropathogen infection.

In conclusion this study allowed us to quantitatively assess the effect of de novo colonization of intestinal commensal bacteria on CD4+ T cell differentiation in vivo using gnotobiotic mice and to establish gene signatures distinguishing regulatory and inflammatory CD4+ T cell subsets based on their induction route. We believe that this knowledge will eventually allow identifying the sources of pathogenic memory cells that alter disease susceptibility upon by-stander activation during inflammatory responses.

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CHAPTER 4

General Discussion and Outlook

The gastrointestinal (GI) tract of vertebrates is densely colonized by complex and dynamic ecosystems consisting of hundreds of species of bacteria as well as archaea, viruses and parasites, commonly referred to as the gut microbiota [1]. These complex ecosystems have coevolved together with the host to maintain a mutualistic partnership [2,3]. Altogether, these microorganisms contribute to various essential physiological functions such as the metabolism of complex dietary ingredients, resistance against colonization of pathogen and proper development of the host's immune system. Specifically, bacteria have been in the focus of numerous studies, since they and their metabolic output represent the largest and most diverse proportion of these communities [4,5]. The advent of culture independent high-throughput DNA sequencing technologies enabled scientists to better classify and characterize the composition of the microbiota. This has led to the realization that in the gut the composition of the microbiota can vary greatly among individuals and it is highly susceptible to change with genetic predisposition or altered environmental factors for instance, diet, antibiotic use, influx or efflux of other microbes [6]. Moreover, diverse disease conditions have been associated with imbalances in the composition of gut microbiota, a state now commonly referred to as dysbiosis [7–9]. While experimental confirmation is still awaited in many pathophysiological conditions, based on findings in a number of diseases it is considered highly probable that differences in community structure and function can actively influence the outcome of many different types of immunological, metabolic and even neurological diseases. Therefore, the understanding of the homeostatic balance in the intestinal microbiota and the crosstalk with the host is crucial to maintain and promote human health.

In the GI tract inflammatory diseases caused by infectious and environmental agents as well as misguided immune responses are the most common forms of GI disorders [10–12]. In the broader sense, the inflammation associated with these conditions is a complex biological response, in which the immune system attempts to neutralize or eliminate injurious stimuli and eventually allow regeneration and healing. Specifically, while the primary role of this inflammatory response is to assist host defense, inflammation can cause substantial collateral damage when over-activated or not properly terminated. Moreover, whether a specific component of the inflammatory response will be beneficial or harmful is dependent on types of stimuli, environmental factors and host immune status. An important part of the inflammatory processes are diverse types of immune cells including different innate immune cells, e.g. macrophage, monocyte, dendritic cells, and neutrophils as well as adaptive immune cells, e.g. T cells and B cells. Different and sometimes overlapping cell subsets secrete immunomodulatory proteins, called cytokines, which are involved in the modulation of inflammation, e.g. interleukin (IL)-1, IL-6, and TNF-α. The important role of some of the cytokines in immune-mediated diseases is demonstrated by improved patient well-being after their neutralization to minimize inflammation. The intestinal microbiota, strongly involved in homeostatic maturation and education of the host immune system has also been associated regulating different inflammatory conditions in GI tract as well as extra-intestinal inflammatory disorders. However, whether microbiota is directly involved in exerting inflammation and whether inflammatory responses modulated by the microbiota are beneficial or harmful remains to be elucidated in detail. Hence, understanding if and how exactly the microbiota modulates intestinal inflammation may help clinicians to develop better and more specific therapeutics for gastrointestinal diseases. The overall aim of the thesis was to characterize the interplay of the hosts immune system with specific model gut microbial communities (chapter 2) and a distinct immunomodulatory commensal (chapter 3) assessing the effect on modulating GI inflammation and to identify distinct *in vivo* host immune response influenced by them.

In the first part of the thesis, we studied a mouse model of inflammatory bowel disease (IBD), which includes a diverse group of immune-mediated diseases in the GI tract. A common characteristic of IBD is damage to the intestinal barrier that during homeostasis serves as critical barrier to separate the intestinal microbiome from the host's immune system [13]. Damage to the intestinal barrier can be modeled in the mouse using the chemical dextran sodium sulphate, which induces barrier damage and subsequently inflammation in response to the microbiota. We demonstrate that alterations in the microbiota result in different susceptibilities to DSS colitis. IBD in human patients has been associated with decrease in overall diversity of the intestinal microbial communities and overgrowth of the phyla Proteobacteria over resident Firmicutes and Bacteroides [14]. Notably, we demonstrated that lowered Firmicutes / Bacteroides ratios occurred in all microbial communities, which were able to enhance colitis susceptibility. Specifically, colitogenic community derived from Nlrp6 inflammasome deficient mice showed lowest ratio of Firmicutes / Proteobacteria. Both innate and adaptive arm of immune system play crucial role to develop IBD [15]. We investigated whether a common theme, lowered Firmicutes / Bacteroides ratios, enhanced colitis development via shared or distinct immune pathways. However, this was not true. Overexpansion of phyla Proteobacteria demonstrated generation of pathogenic CD4+ T cells with ability to secret distinct proinflammatory cytokines i.e. IL-17A and IFNγ. In contrast, another colitogenic community only with over expanded Bacteroides phyla could develop colitis already in absence of adaptive immunity and demonstrated significant infiltration of neutrophils and associated chemokines and higher TNFα. Therapeutic strategies targeting proinflammatory cytokines are commonly used in IBD patients [16]. However, anti-cytokine therapies (such as antibodies specific for TNF, IL-12 or IL-23) and cytokine signaling blockers (such as tofacitinib) only seem to have beneficial clinical effects in certain subgroups of patients [17–19]. Variability in microbiota composition among patients may reflect such differences in efficacy of therapies. Our data suggesting a microbiota dependent pathway of disease development indicate careful investigation of IBD patients to identify specific microbiota communities or microbiota-induced

immune effectors as biomarkers may help clinicians to develop personalized IBD therapy.

Targeting or manipulating a broad intestinal microbial community may disrupt normal homeostatic balance. Therefore, identifying single bacterial species modulating specific host immune pathway involved in enhancing or mitigating inflammation may be associated with less unwanted side-effects. We hence extended our investigation to characterize the effect of specific intestinal microbial species on the immune system. We focused on segmented filamentous bacteria (SFB), a commensal present in both colitogenic communities. SFB intimately attaches to the intestinal epithelial cells and is known to induce distinct host immune responses including IgA [20], Th17 cells [21] and Treg [22] induction. In different models of intestinal and extra-intestinal immune-mediated inflammatory diseases SFB has been reported to exacerbate inflammation [23–25]. However, SFB has also shown to provide enhanced resistance against intestinal inflammation caused by *Citrobacterium rodentium* [21]. We therefore wanted to investigate whether SFB alone can exert beneficial *in vivo* host immune response and protection in models of intestinal enteropathogen infection. Using a mouse model of *Salmonella* induced gastroenteritis we demonstrated that SFB alone could specifically induce distinct subsets of CD4+ T cells with the ability to secret multiple immunomodulatory cytokines, i.e. IL-17A, IL-17F, IL-22 and IFNγ. These cytokines have beneficial effects of providing protection against infection via multiple pathways i.e. enhanced antimicrobial peptide secretion, tissue regeneration, increased bacterial killing. Indeed, SFB colonization in our model enhanced colonization resistance against *Salmonella* invasion. However, whether interaction of SFB with other intestinal components of the microbiota can exert synergistic protective effects needs to be investigated in future.

Mouse model of human disease are powerful tool to investigate role of host immunity and microbiota during both homeostasis and inflammation. However, presence of certain variables in the experimental system can affect

reproducibility and interpretation of the results. Studies have shown that microbiota composition in the same mouse lines from different facilities [26–28], or even within one facility [29] can vary significantly and such variability has resulted in different outcomes. As an example, possible contradictions in studies on Toll-like receptor 5 (TLR5) influencing intestinal microbiota compositions were largely due to husbandry history rather than genotype [30]. Hence, throughout our entire study we have undertaken careful approach to maintain standardized experimental systems to avoid unwanted effects of different variables like genotype, environmental factors etc on the murine microbiota. Specifically, researchers have focused to obtain standardized microbiota models lacking potential pathobionts and then further manipulate the microbiota through e.g. cohousing or cross fostering. In this study we have employed standardized isobiotic mouse lines in which the microbiota could be manipulated at will. Moreover, we ensured genetic background and use of germ free mice and cohousing also allowed us to further confirm microbiota effect. However, to study direct effect of human microbiota on host immunity, besides association studies more sophisticated techniques like humanized mouse models need to be employed.

In conclusion, together our studies strongly support the fact that in an immune-competent host alteration in microbiota composition alone can drastically influence disease pathogenicity. Distinct members of microbial species are able to induce CD4+ T cells, however such microbiota modulated T cell functionality can be both beneficial or harmful depending on inflammatory conditions. Further studies are needed to identify specific microbial members inducing pathogenic and beneficial T cell responses in host and whether distinct T cell subsets inducing bacterial species can interact with each other to influence disease outcome. Moreover, detailed metabolic profiling of such immunomodulatory microbiotal species may reveal distinct metabolites driving such induction of pathogenic or beneficial T cell responses. This may help to target or add certain metabolites during inflammatory diseases to obtain similar effects exerted by the microbiota itself.

Most importantly, similar microbiota induced effects are most likely to be also present in human and detailed careful investigation of such complex interaction in human subjects may help to identify specific immunomodulatory responses provided by human microbiota. Which in turn may help to stratify patients according to their microbiotal composition and imply to develop personalized therapies targeting microbiota or microbiota induced host responses against different inflammatory diseases.

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Curriculum Vitae

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- Animal experimentation, cell culture techniques, bacteria culturing related techniques
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- Career and Leadership- Making Powerful Choices and a Positive Impact
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PUBLICATIONS

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